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Growth and Nitrogen Fixation of *Lotus japonicus* Under Simulated Martian Soil- and Atmospheric Conditions



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Front image generated by Miel Houtenbos

With ChatGPT: Generate an image on A4 of the Martian surface with at the center a Legume plant coming out of the ground and a white horizontal bar in the middle. Use orange vibrant colors and a schematic, infographic style



Growth and Nitrogen Fixation of *Lotus japonicus* Under Simulated Martian Regolith and Atmospheric Conditions

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Abstract

As human missions to Mars move closer to reality, the question arises whether plants can be cultivated using only Martian resources. This study investigates whether *Lotus japonicus*, a model legume species, can grow and survive on simulated Martian soil and under Mars-like atmospheric conditions, and whether nitrogen-fixing rhizobacteria can enhance its growth under these stresses. Plants were cultivated on two substrates: the Mojave Mars Simulant (MMS-2), representing Martian regolith, and nutrient-free quartz sand as a control. Each substrate was tested both with and without inoculation using *Mesorhizobium sp. LjRoot218*.

Surprisingly, *L. japonicus* grew significantly better on MMS-2 than on quartz sand, showing increased shoot length, root development, and dry biomass. This was unexpected given the extreme alkalinity of MMS-2 (pH 10–11), which exceeds previously reported Martian regolith pH values (~8) and is known to impair nutrient availability and symbiosis. Despite these favorable growth results, *Mesorhizobium* inoculation showed little effect: nodulation was rare, and no significant differences in growth were observed between inoculated and uninoculated plants, suggesting that effective nitrogen-fixing symbiosis was likely limited or unsuccessful.

In parallel, soil chemistry experiments revealed that MMS-2 strongly resists acidification, with acid and buffer treatments failing to substantially lower pH. This suggests that correcting soil pH on Mars may prove difficult without significant technological intervention. Finally, seed germination under a low-pressure, Mars-like atmosphere (300 mbar) was tested using a modified Miller-Urey chamber. Although germination rates declined under these conditions, 25% of seeds still germinated successfully.

These results show that *L. japonicus* can tolerate several harsh Martian soil and atmospheric conditions, though challenges remain for establishing effective microbial symbiosis and managing extreme soil pH. This study provides valuable first steps toward assessing the potential for crop cultivation in extraterrestrial environments.

Key words: Astrobiology · *Lotus japonicus* · Martian soil simulant · MMS-2 · Nitrogen fixation · *Rhizobium* symbiosis · soil pH buffering · Low-pressure germination · Miller-Urey

Introduction

Following the historic landings of the Perseverance and Tianwen-1 rovers on Mars in 2021, humanity's next frontier in space exploration is the successful landing and survival of humans on the Martian surface. However, once humans are sent to Mars, there is currently no feasible way to bring them back, making it essential for long-term missions to be fully self-sustaining. A key part of this is food production. Supplying metabolic consumables for a six-member crew on a 1,000-day mission would require more than 30 metric tons of food and 102 metric tons of clean water (Maity & Saxena, 2024). This introduces an immense logistical challenge of crop selection and a necessity to retrieve the maximum amount of nutrients available on the planet (Langhoff et al., 2011). This raises the central question: To what extent can plants grow, develop, and sustain themselves using only the limited resources available on Mars? Achieving this would require crops capable of surviving multiple environmental stressors simultaneously — including extreme nutrient limitations, poor nitrogen availability, high pH levels, and a vastly different atmospheric composition.

To date, no physical Martian soil has been returned to Earth, so most studies rely on in situ rover data and orbital spectroscopy to infer the planet's surface composition (Bandfield et al., 2000). These missions have revealed that the planet's regolith, a sand-like material covering the surface, contains most of the essential elements required for plant growth, including macro elements (C, H, O, N, P, S, K, Mg, Na and Ca) and minor metals (Mn, Cr, Ni, Mo, Cu, Fe and Zn) (Kasiviswanathan et al., 2022; Wamelink et al., 2014). However, most of these elements occur as oxides such as SiO_2 , Fe_2O_3 and CaO (table 1), which are poorly soluble in water and not readily available to plants (Kasiviswanathan et al., 2022). Furthermore, nitrogen is mostly present in unreactive forms (N_2 or nitrides), not as nitrate (NO_3^-) or ammonium (NH_4^+), which is an essential mineral for plants, being a key component of chlorophyll which enables the plant to get energy and grow by photosynthesis (Wamelink et al., 2019). On Earth, reactive nitrogen forms are generated through volcanic activity, lightning, and, most importantly, biological nitrogen fixation by microbes (Glass, 2003), but such processes are either absent or highly limited on Mars due to its thin atmosphere (only 0.6% of Earth's) and lack of active plate tectonics (Glass, 2003).

Substrate composition in percentage			
Oxide	MMS-2	Mars average	Quartz sand
SiO ₂	43.80	43.52	99.50
Fe ₂ O ₃	18.37	18.28	
Al ₂ O ₃	13.07	8.64	
CaO	7.98	6.09	
MgO	6.66	6.54	
SO ₃	6.11	6.42	
Na ₂ O	2.51	2.57	
P ₂ O ₅	0.13	0.79	
TiO ₂	0.83	0.78	
K ₂ O	0.37	0.35	
MnO	0.13	0.32	
Cr ₂ O ₃	0.04	0.37	
Total:	100.00	94.67	99.5

Table 1. Composition of substrates (in percentage) used in the study: Mojave Mars Simulant 2 (MMS-2), average Martian regolith composition, and sterile quartz sand. All present oxides are listed, with MMS-2 closely matching Martian regolith but lacking perchlorates. Quartz sand serves as nutrient-free control.

One possible solution is to introduce nitrogen-fixing rhizobacteria alongside crops. On Earth, these bacteria form symbiotic relationships with legume roots, leading to the development of specialized structures called nodules where atmospheric nitrogen (N₂) is enzymatically fixed into ammonia (NH₃), a form directly available to plants. This allows legumes to thrive even in soils with limited reactive nitrogen, which would otherwise severely restrict development as nitrogen is an important element for plant growth and the formation of chlorophyll necessary for photosynthesis. In nutrient-poor substrates like quartz sand, where virtually no available nitrogen is present, such symbiosis may fully compensate for nitrogen deficiency and sustain plant development. In Martian regolith simulants like MMS-2, nitrogen-fixing rhizobacteria could in theory serve the same function. However, beyond nitrogen fixation, these bacteria may provide additional benefits critical in alien soils, such as improving phosphorus availability through organic acid secretion and enhancing iron uptake via siderophore production (Ahemad & Kibret, 2014; Clark et al., 2021). These additional traits may help support plant nutrition under broader Martian chemical limitations.

In this study, I use *Lotus japonicus*, a model legume species widely used in symbiosis research due to its short life cycle, well-characterized genome, and established compatibility with rhizobial strains such as *Mesorhizobium*. Its ability to form root nodules makes it an ideal candidate to study symbiotic nitrogen fixation in alien soils. However, this strategy faces complications on Mars. Phosphorus availability, for instance, drops sharply in alkaline soils, a property observed in Martian soil simulants. In fact, while Martian soil pH values are reported around ~8 (Sutter, 2021), this study found the pH of the Mojave Mars Simulant (MMS-2) to be significantly higher at ~10–11. These conditions are especially problematic for legumes such as *L. japonicus*, which require mildly acidic to neutral pH (~6.4) for successful symbiosis with

rhizobia (Bordeleau & Prévost, 1994). While low phosphorus availability is known to directly hinder *Rhizobium*-Legume symbiosis (Martins et al., 2017).

To understand the feasibility of agriculture on Mars, several simulated Martian regoliths have been developed. MMS-2, designed by NASA's Jet Propulsion Laboratory, is one of the most widely used. While it lacks Martian-specific compounds like perchlorates, which are present on Mars and known to be phytotoxic (Eichler et al., 2022), MMS-2 accurately represents Martian soil in terms of particle size, bulk density, and oxide composition. As a nutrient-free baseline comparison, sterile quartz sand was used in the plant growth experiment. This choice was based on its inclusion in earlier Martian regolith studies (e.g., Rainwater & Mukherjee, 2021) and its chemically inert nature. Composed of over 99% SiO₂ (table 1), quartz sand contains no bioavailable nutrients, making it a candidate reference point to assess nutrient properties of MMS-2. Its use allows direct evaluation of whether the metal oxides and trace elements present in Martian regolith simulants can independently support plant growth, even in the absence of organic matter. Prior studies using MMS-2 and other simulants have highlighted the harshness of Martian conditions but have also shown that both plants and soil bacteria can survive for a prolonged time given the right atmospheric conditions (Wamelink et al., 2014; Rainwater & Mukherjee, 2021).

In addition to soil chemistry, the Martian atmosphere poses unique physiological challenges. While Mars has an atmospheric CO₂ concentration of about 95%, far higher than Earth's 0.04%, it lacks adequate nitrogen and water vapor and exists at only 0.6% of Earth's atmospheric pressure. Although increased CO₂ is known to stimulate plant growth under greenhouse conditions (Hender, 2007), little is known about how seeds or microbes respond to the low-pressure and gas composition of Mars. To address this, the current study includes an experimental component in which seeds are germinated in a modified Miller-Urey apparatus, a closed-loop glass system originally developed to simulate early Earth conditions. Here, the chamber has been adapted to mimic a mixed Earth-Mars atmosphere at a pressure of 300 mbar, consisting of 60% N₂, 20% O₂, and 20% CO₂. While this experiment does not fully replicate Martian atmospheric conditions, it provides a controlled, low-pressure analog to investigate seed germination under partially Mars-relevant environmental stresses, which has not been addressed in the consulted research and is therefore groundbreaking in this aspect.

This research therefore aims to investigate the capacity of *L. japonicus* to grow in Mars soil simulants and assess how key environmental stressors, like high pH, nutrient limitation, and non-Earth-like atmospheres, affect its development and survival. It will also evaluate whether inoculation with *Mesorhizobium* improves plant growth under these conditions. Taken together, these experiments will evaluate the boundaries of plant resilience in Martian analog environments.

This study consisted of three interconnected experiments. First, a plant growth experiment assessed the development of the legume *L. japonicus* grown on the Martian soil simulant MMS-

2 and a nutrient-free soil composed of sterile 0.5–1.0 mm quartz sand. Both treatments were inoculated with and without the nitrogen-fixing bacterial strain *Mesorhizobium* sp. *LjRoot218*. All treatments were maintained under controlled conditions in a climate chamber, where shoot growth was monitored every other day. At the end of the growth period, plants were harvested for detailed analysis, including measurements of shoot and root tissues and examination of root nodules as indicators of successful symbiosis.

Second, an exploratory soil chemistry experiment was conducted to evaluate the buffering capacity of MMS-2 and test whether its highly alkaline pH could be reduced to more closely resemble the optimal range for *L. japonicus* nodulation and growth.

Finally, a third experiment investigated seed germination under atmospheric stress. Seeds were germinated on MMS-2 under Earth atmospheric conditions and within a modified Miller-Urey setup designed to simulate a Mars-like atmosphere at low pressure. This experiment helped assess the resilience of *L. japonicus* during early developmental stages under extraterrestrial conditions.

It was expected that *L. japonicus* would show reduced growth on MMS-2 compared to nutrient-free sand due to limited nutrient availability and high pH. Inoculation with *Mesorhizobium* was expected to improve plant growth and nodulation on MMS-2 by compensating for nitrogen limitations. The alkaline pH of MMS-2 was hypothesized to lower plant health and development, so individuals grown on MMS-2 with a lowered pH (6.4) were expected to show increased growth and development. Finally, a lower germination success for *L. japonicus* seeds was expected under low-pressure Mars-like atmospheric conditions compared to Earth atmosphere.

This study is novel in multiple respects. To the best of my knowledge, no previous experiment has evaluated legume seed germination in a controlled low pressure atmosphere within a Miller-Urey-type system. Beyond its scientific contributions, this research holds significant societal relevance. As humanity moves closer to the reality of interplanetary colonization, the ability to grow crops on Mars will be essential for the long-term sustainability of human missions. Developing methods to cultivate plants in extraterrestrial environments not only advances our understanding of plant biology under extreme conditions but also has potential applications on Earth. The insights gained from this study could inform strategies for improving crop resilience in arid or nutrient poor soils here on earth, addressing food security challenges in the face of climate change. By bridging the gap between space exploration and terrestrial agriculture, this research contributes to both the future of space colonization and the pressing need for sustainable farming practices on Earth.

Method

Plant growth analysis

Experimental design

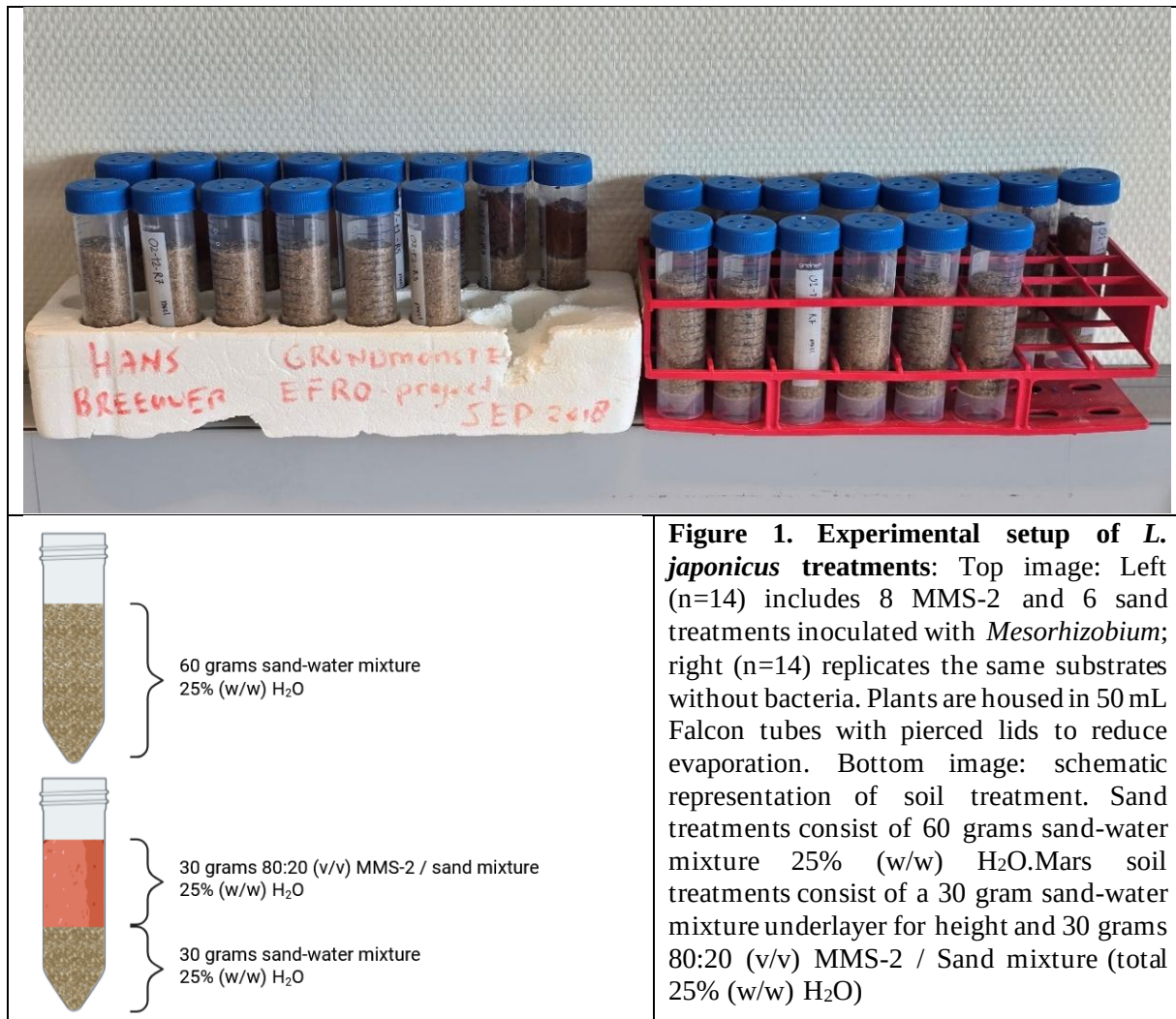
This study consisted of three interconnected experiments to evaluate the effects of nutrient availability, nitrogen fixation, pH buffering, and atmospheric stress on the growth and germination of *Lotus japonicus*. Two soil substrates were prepared: (1) an 80:20 (w/w) mixture of Mars regolith simulant MMS-2 superfine (1.37 g/cm³; The Martian Garden) and autoclaved quartz sand (1.5 g/cm³, 0.5–1.0 mm, pH-neutral), and (2) 100% quartz sand as a nutrient-free baseline control. Quartz sand was autoclaved (140°C, 2.5 h) prior to use and hydrated with 25% (w/w) sterile water. The MMS-2 substrate was not autoclaved due to its superfine structure, which would consolidate when in contact with water and make soil preparation difficult. Following soil preparation, plants were grown with or without *Mesorhizobium* inoculation to assess nitrogen fixation effects.

Seed sterilization and germination

L. japonicus seeds were scarified by thumb using P120-grit sandpaper, applying medium force until the seed coat appeared glossy. Surface sterilization was then performed by agitation in 10 mL sterile water containing 1 mL sodium hypochlorite (<2.5% active chlorine) for 20 min at 185 rpm. The seeds were rinsed five times in a flow chamber before being transferred onto autoclaved, moistened Whatman paper (grade 3) in square Petri dishes (12 × 12 cm) at ±1 cm spacing. The dishes were sealed with two layers of parafilm and germinated under controlled conditions (25°C, 55% RH, 16-h photoperiod, 4LS light intensity) for three days (Appendix, protocol “Sterilization and germination of *L. japonicus* seeds”).

Planting and inoculation

For each treatment, 50 mL Falcon tubes were filled as follows: MMS-2 tubes (n = 16) contained a 30 g base layer of sand-water mixture topped with 30 g of MMS-2/sand mixture. Sand-only tubes (n = 12) were filled with 60 g of sand containing 25% autoclaved tap water. After filling, two 5 mm holes were dug with consistent spacing, and two seedlings were transferred into each tube. After eight days, plants were thinned to one per tube to maximize initial acclimation and reduce competition. Lids were punctured with 4 ± 2 mm holes to minimize evaporation in the climate chamber. On day 8, bacterial inoculation was performed during watering by applying 500 µL of *Mesorhizobium* suspension (OD₆₀₀ 0.2, prepared as described below) directly to the substrate surface of halve MMS-2 and sand treatments (n = 8 for MMS-2 and n = 6 for Sand) (figure 1).



Mesorhizobium culture preparation

Bacterial cultures from the strain *Mesorhizobium* sp. *LjR218* were grown in tryptone yeast (TY) medium, prepared by dissolving 2.5 g tryptone (Duchefa, T1332.1000) and 1.5 g yeast extract (Duchefa, Y1333.1000) in 500 mL Milli-Q water. For solid medium, 7.5 g Daishin agar (Duchefa, D1004.1000) was added. Both media were autoclaved (140°C, 1.5 h), followed by the addition of 5 mL sterile 1 M CaCl₂. Media preparation was performed under sterile conditions in a flow chamber (Appendix, protocol “TY growth medium for *Mesorhizobium*”).

Solid TY medium was poured into agar plates (n = 4), including one sterile control and three plates streaked with *Mesorhizobium* *LjR218*. The original plate consisted of a few large overlapping colonies (Figure 2). Due to the colony size, distinct colonies could not be isolated, and streaks from the large colonies were used for further culturing. Plates were incubated at 27°C for 96 h.

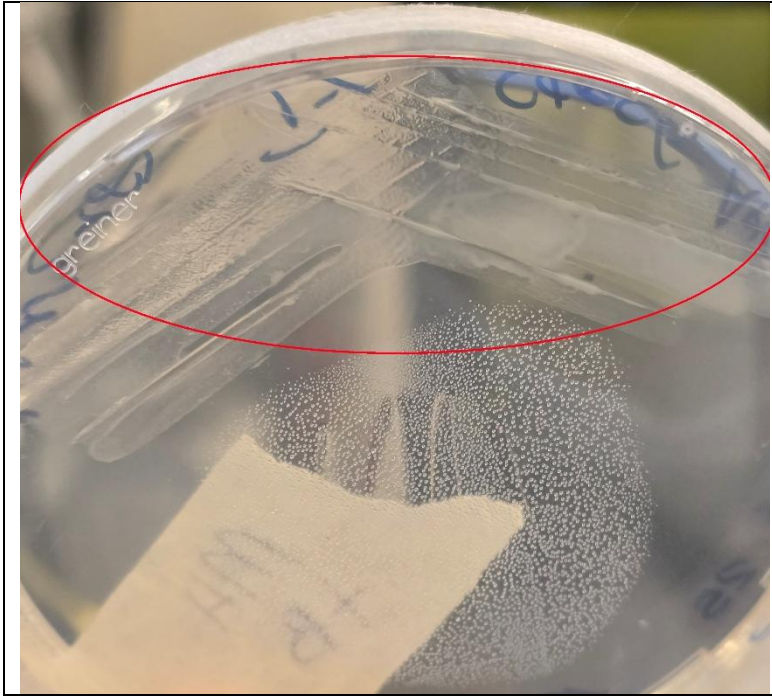


Figure 2. Pre-inoculated TY agar plate with *Mesorhizobium* LjR218 streaks: The bottom streak (condensed area above the tape) shows no bacterial growth, while the top streaks (red circle) show dense, cloudy bacterial plaques without distinct colonies.

For liquid cultures, four 15 mL Falcon tubes containing 2 mL liquid TY medium were inoculated: one sterile control and three with bacterial streaks from plates. These were incubated at 25°C with shaking (185 rpm) for 24 h. After this initial period, multiple dilutions were prepared to time bacterial growth with inoculation. 1 mL of each culture was transferred into 15 mL Falcon tubes containing either 2 mL (for 3 mL cultures) or 9 mL (for 10 mL cultures) of fresh TY medium. All cultures were incubated (25°C, 185 rpm, 72 h), and OD600 was measured to assess growth. Cultures reaching OD600 0.3–0.4 were cold-shocked on ice and stored at 4°C until inoculation. Cultures with OD600 > 0.4 were diluted to the target density, while cultures with OD600 < 0.3 were incubated for an additional 72 h. On inoculation day, refrigerated cultures still showed marginal growth. One sample was pelleted (3,500 rpm, 5 min), washed with 3 mL 10 mM MgSO₄, and resuspended to an OD600 of 0.2. This concentration was chosen to minimize potential stress from excessive bacterial load (Appendix, protocols “Bacterial inoculation”).

Plant growth conditions and morphophysiological measurements

The plant samples were grown in a climate chamber (25°C, 55% RH, 16-h light at 4LS) and watered with 1 mL tap water every 48 h (apart from weekends and mandatory days off). Before watering, the samples were taken out of the climate chamber for visual analysis. A tweezer was used to keep the plants upright while the length was documented with a thin ruler. The plants were subsequently watered and brought back to the climate chamber. After 30 days, roots were

gently rinsed free of substrate using sterile water. After the roots were freed, the plants were transferred to a petri dish with water to wash the roots off with a soft brush. The plants were then transferred to tissue paper and blotted dry.

Primary and lateral root/shoot lengths (mm) and lateral root/shoot counts were recorded. Nodulation (counts, color: pink = active fixation; yellow/transparent = inactive) and biomass (fresh/dry weight) were quantified. Fresh weight was recorded by cutting the plants at the root and immediately measuring weight of both root and shoot on a laboratory scale. Dry weight was measured the same way after drying the plants for 96 h at 60 degrees in a drying oven in Falcon tubes (lid loose).

Soil pH analysis

An additional experiment evaluated whether MMS-2 pH could be adjusted toward optimal values for *L. japonicus* growth (~pH 6.4). Initial pH of MMS-2 was measured by suspending 13.7 g in 20 mL tap water (2:1 v/v) in a 50 mL Falcon tube. The suspension was shaken vigorously for 5 minutes and then vented for 1.5 h (lid open) before measurement using a calibrated Mettler Toledo S220 pH meter (standards: pH 4.03, 7.00, 9.01) (figure 3).

To lower pH, 2 M HCl was incrementally added (total 5.1 mL), but only modest pH reductions were observed. Due to the soil's high buffering capacity, a low-pH stabilization buffer was introduced using 1 M MES (2-(N-morpholino)ethanesulfonic acid, pH 5.6). Dilutions (0.1 M and 0.2 M MES) were prepared by mixing 4 mL or 8 mL of 1 M MES with 36 mL and 32 mL Milli-Q, respectively. Fresh MMS-2 samples (13.7 g) were mixed with these buffers (2:1 v/v), vortexed (10 sec), and measured after clarification (~10 min) and at 24/48 h intervals. Due to the still high pH of the soil, acid titration was performed on a new sample of 3.0 g MMS-2 in 15 mL water (1:5 w/v). 2 M HCl was added in 400 μ L increments to the mixture, with 10-sec vortex mixing after each addition and 5-min settling before each measurement. Due to time constraints and still unknown pH characteristics of the regolith the pH treatment on *L. japonicus* was removed.

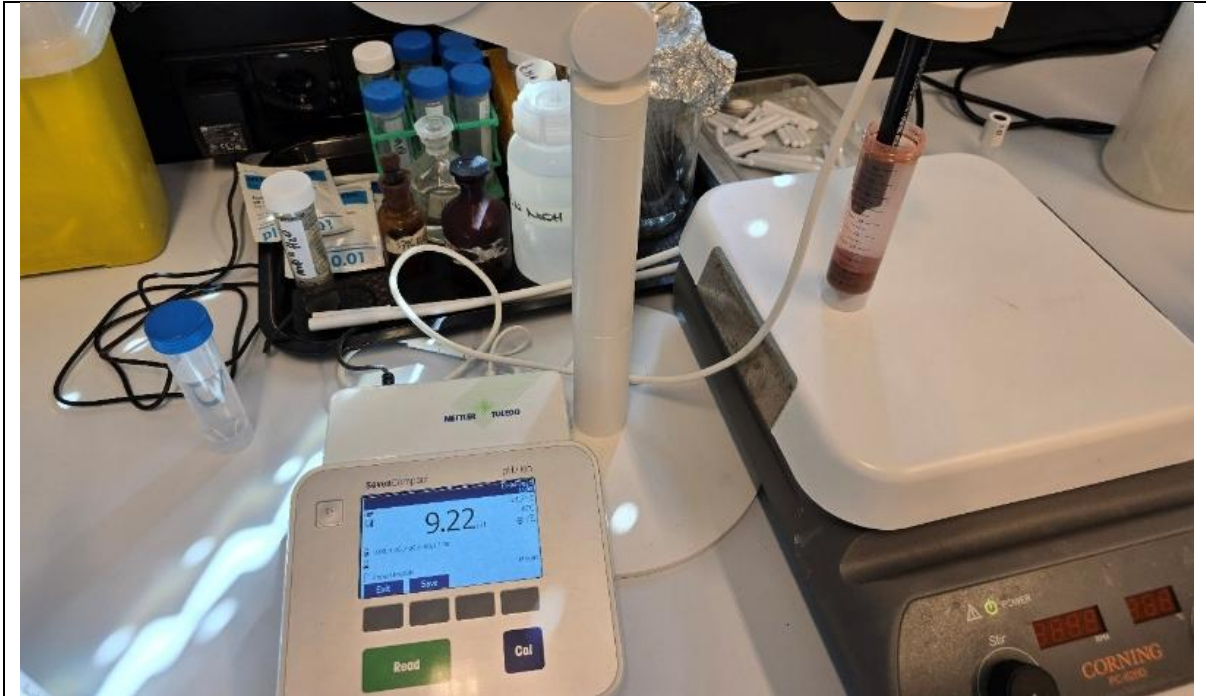


Figure 3. pH measurement setup using a Mettler Toledo S220 pH meter. A 50 mL Falcon tube contains MMS-2 regolith suspended in autoclaved tap water (2:1 v/v) after settling for 1.5 hours. pH on display not starting value.

Seed germination in Miller-Urey system

The germination capacity of *L. japonicus* seeds under simulated Martian atmospheric conditions was evaluated using a modified Miller-Urey reaction chamber system (figure 4). Surface sterilized seeds ($n = 20$ per treatment) were subjected to three experimental conditions: (1) a sterile control consisting of Whatman paper moistened with 5 mL autoclaved water, (2) a substrate control using 60 g of MMS-2 regolith hydrated to 35% water content (w/w), and (3) an experimental group containing identical hydrated MMS-2 within a 3,500 mL Miller-Urey vessel under simulated hybrid Martian-Earth atmosphere.

The atmospheric simulation was achieved by first establishing a vacuum base of 13 mbar in the 3500 mL flask with a vacuum pump, followed by sequential gas injection of 60 mbar CO_2 , 141 mbar nitrogen gas (N_2), and 105 mbar compressed air (as an O_2 source), resulting in a final internal pressure of 319 mbar. This mixture approximated a 70:30 Earth:Mars gas proportion ratio, at 1/3 earth sea level pressure (table 2). The potential contribution of water vapor to the chamber atmosphere, resulting from the hydrated regolith, was not quantified. Both petri dishes were sealed with Parafilm, and all treatments were maintained at 20°C under ambient LED lighting and assessed after 7 days.

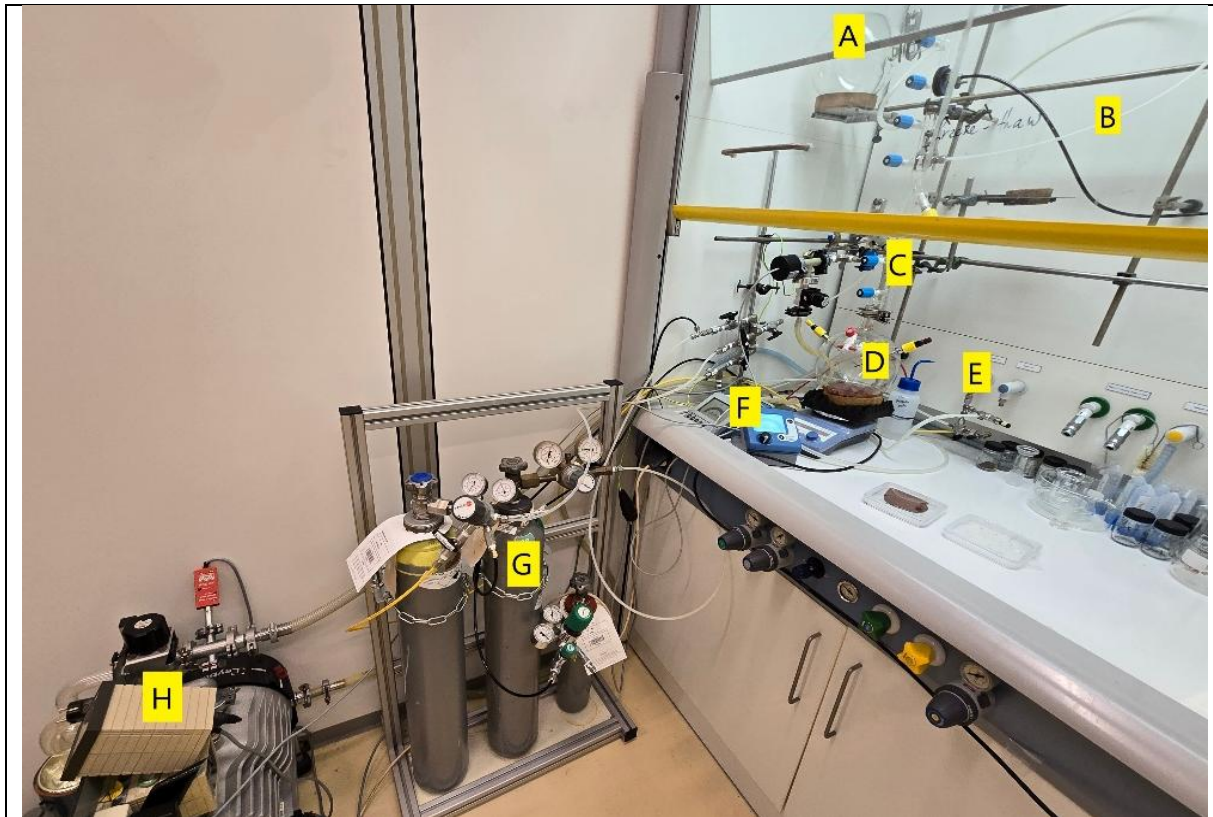


Figure 4. Modified Miller-Urey setup for simulating atmospheric conditions: Components: (A) Reactor flask with gas mixture, (B) N₂ gas tube, (C) Vacuum manifold, (D) Flask with MMS-2 and seeds, (E) Compressed air valve, (F) Barometer, (G) Liquid CO₂ tank, (H) Vacuum pump.

Atmospheric gas	Earth (Sea Level)	Mars	70:30 Earth:Mars	Simulated atmosphere
Total Pressure	1,013 mbar	6.36 mbar	711 mbar	312 mbar
N ₂	78.1% (791 mbar)	2.7% (0.2 mbar)	77.9% (553.8 mbar)	44.9% (140 mbar)
O ₂	21% (212 mbar)	0.1% (0.008 mbar)	20.9% (148.4 mbar)	6.7% (Air)(21 mbar)
CO ₂	0.04% (0.4 mbar)	95.3% (6.1 mbar)	0.3% (2.1 mbar)	19.2% (60 mbar)
Ar	0.94% (9.4 mbar)	1.6% (0.1 mbar)	0.9% (6.6 mbar)	1.3% (4 mbar)

Table 2: Comparative atmospheric gas compositions (pressure and partial pressures) for: Earth (sea level), Mars, a theoretical 70:30 Earth-Mars hybrid, and the simulated atmosphere used in the Miller-Urey experiment (319 mbar total pressure).

Statistical analysis

Growth metrics (shoot length, root length, lateral root count, biomass) were compared across substrates (MMS-2 vs. sand) and inoculation treatments ($\pm$ *Mesorhizobium*) using two-way ANOVA followed by Tukey's HSD post-hoc tests ($\alpha = 0.05$). Assumptions of normality and homogeneity of variance were verified via Shapiro-Wilk and Levene's tests, respectively. All statistical analyses were performed in R v4.3.1 (R Core Team, 2023).

Results

Plant growth Dynamics

Growth Performance Across Substrates

The first hypothesis posited that *L. japonicus* would exhibit reduced growth on MMS-2 compared to quartz sand due to nutrient limitations and high pH. Contrary to this expectation, plants grown in MMS-2 displayed significantly greater shoot elongation, root development, and biomass accumulation than those in sand (Figures 5–6). Shoot lengths in MMS-2 averaged 21.9 ± 4.7 mm (uninoculated) and 21.3 ± 6.7 mm (inoculated), while sand-grown plants stagnated at 8.3 ± 1.5 mm and 8.0 ± 2.5 mm, respectively (Games-Howell post-hoc, $p < 0.0001$). There was no difference found between soil type and bacterial inoculation (Two sample t-test $p > 0.83$)

Root systems followed a similar trend: MMS-2 plants developed primary roots four times longer (44.2 ± 8.1 mm) than sand-grown plants (10.0 ± 2.7 mm; Dunn's test, $p < 0.001$), with uninoculated MMS-2 plants outperforming inoculated ones (49.5 mm vs. 39.0 mm; t-test, $p < 0.005$). Lateral root proliferation was also twice as high in MMS-2 (5.4 ± 2.6 vs. 2.6 ± 1.2 in sand; Dunn's test, $p < 0.005$).

Shoot growth analysis over time revealed distinct developmental trajectories between the treatments (Figure 5). MMS-2 treatment plants maintained consistent linear growth throughout the experimental period, typically gaining between 5-10 mm in shoot length after day 13. Sand treatments however, reached their maximum shoot length by day 15 before either stagnating or showing signs of regression, with some individuals losing up to 2 mm in shoot length by harvest. All individuals showed yellowing of the leaves early during development which remained or increased during the entire growth period (Data repository "Plant_growthvertime_table"). During day 25-28 the climate chamber accidentally turned off resulting in more intense discoloration in the leaves of all plants.

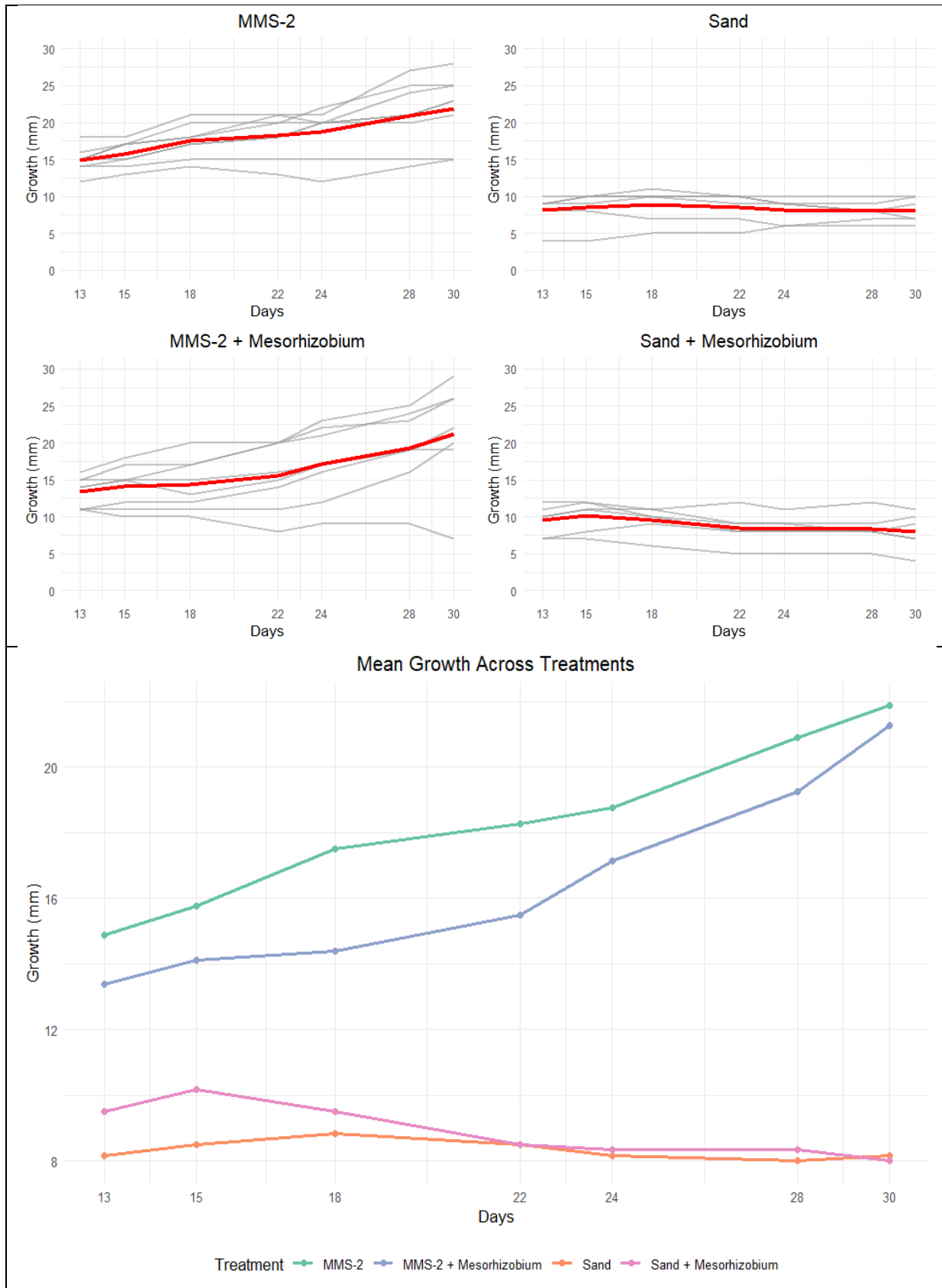
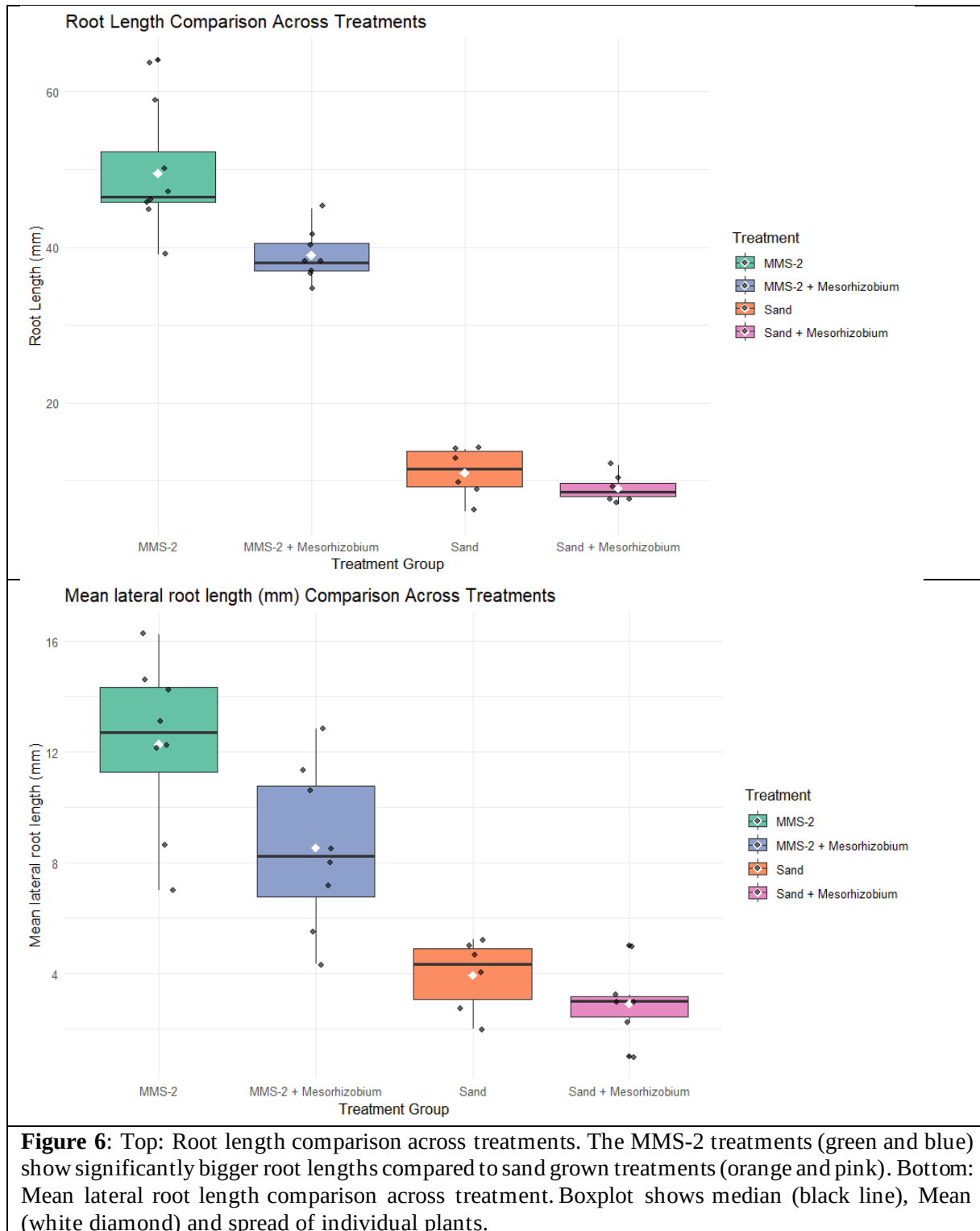


Figure 5: From top: Shoot growth dynamics over time of *L. japonicus* Individual replicates (grey lines) and mean growth (red line) for 30 days MMS-2 regolith samples show upward trend and sand samples downward. Bottom: Mean growth (red lines) for all treatments shown in same graph. Replicates in MMS-2 regolith show clear enhanced growth compared to sand replicates.

Root- length Nodulation and Biomass

The root systems of *L. japonicus* also showed strong differences between substrates for both primary and lateral growth (Figure 6). Plants grown in MMS-2 developed long root systems (44.2 ± 8.1 mm) four times longer than sand-grown individuals (10.0 ± 2.7 mm). Statistical analysis confirmed this substantial difference (Dunn's test with Bonferroni correction, $p < 0.001$). MMS-2 grown individuals without *Mesorhizobium* displayed significantly longer root systems compared to inoculated individuals (49.5mm v 39.0 mm) (t-test $p < 0.005$). This disparity was not found with sand individuals (t-test $p > 0.20$).

Plants grown in MMS-2 also developed significantly more lateral roots than those in sand (Dunn's test, $p < 0.005$). On average MMS-2 plants had twice as many lateral roots (5.4 ± 2.6 lateral roots per plant in MMS-2 vs 2.6 ± 1.2 in sand). MMS-2 grown individuals without *Mesorhizobium* also displayed significantly more lateral roots compared to inoculated individuals (t-test $p < 0.02$). This disparity was not found with sand individuals (t-test $p > 0.80$).



Five inoculated individuals (four in MMS-2 and one in sand) developed nodules, confirming limited but detectable bacterial activity (figure 7). All four nodules formed on the MMS-2 plants were similar in shape and size, and they were transparent red because of the soil the roots were in. The nodule on the sand individual was a darker yellow, softer, and relatively bigger. All nodulated plants displayed only one nodule in the root system.

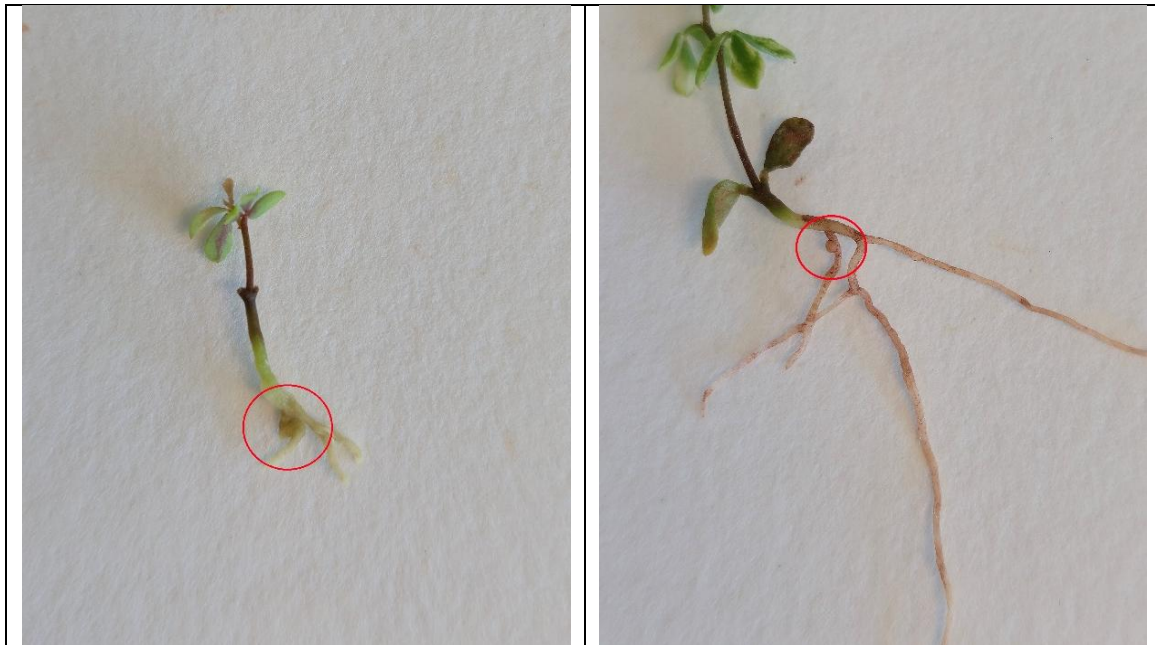
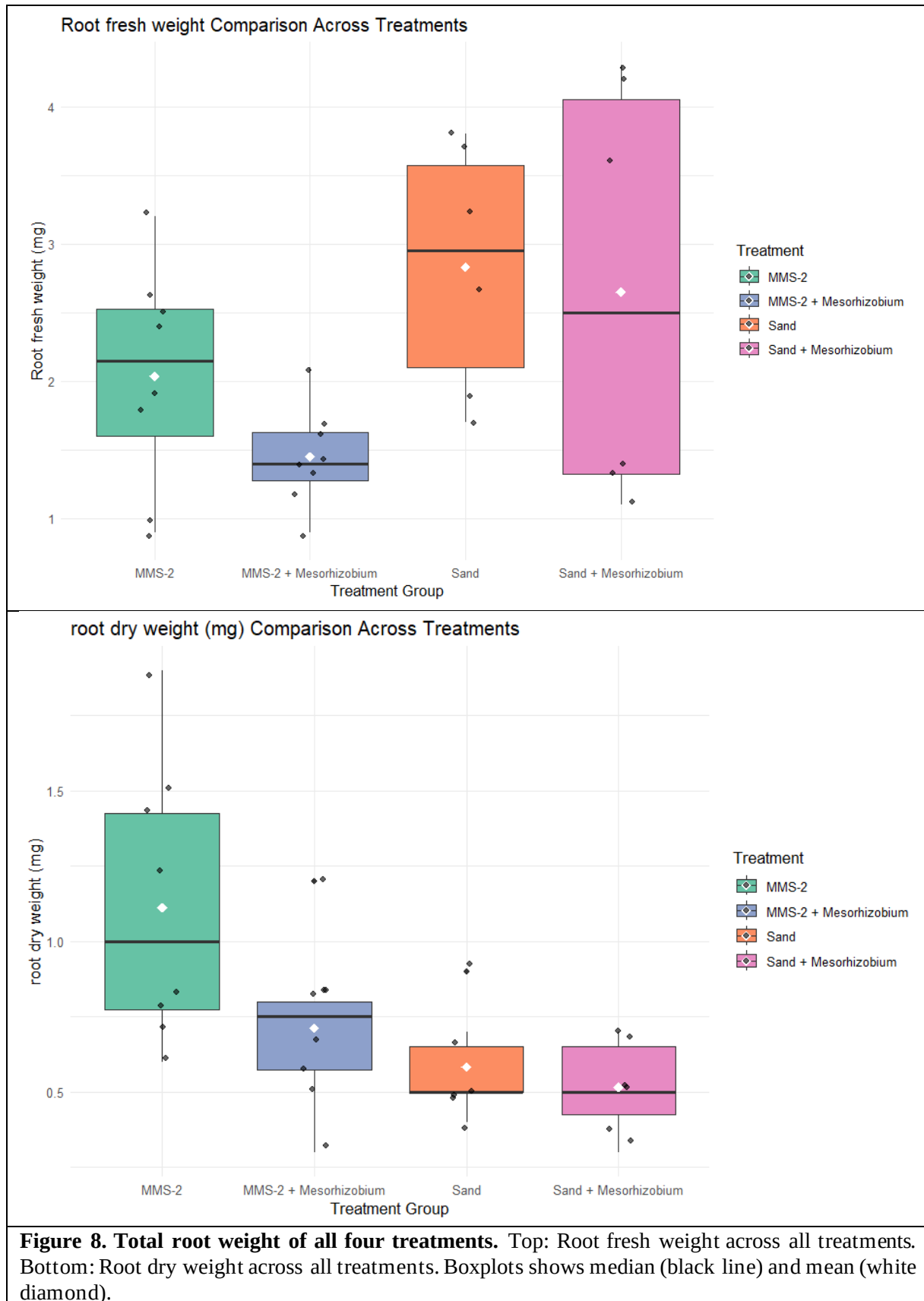


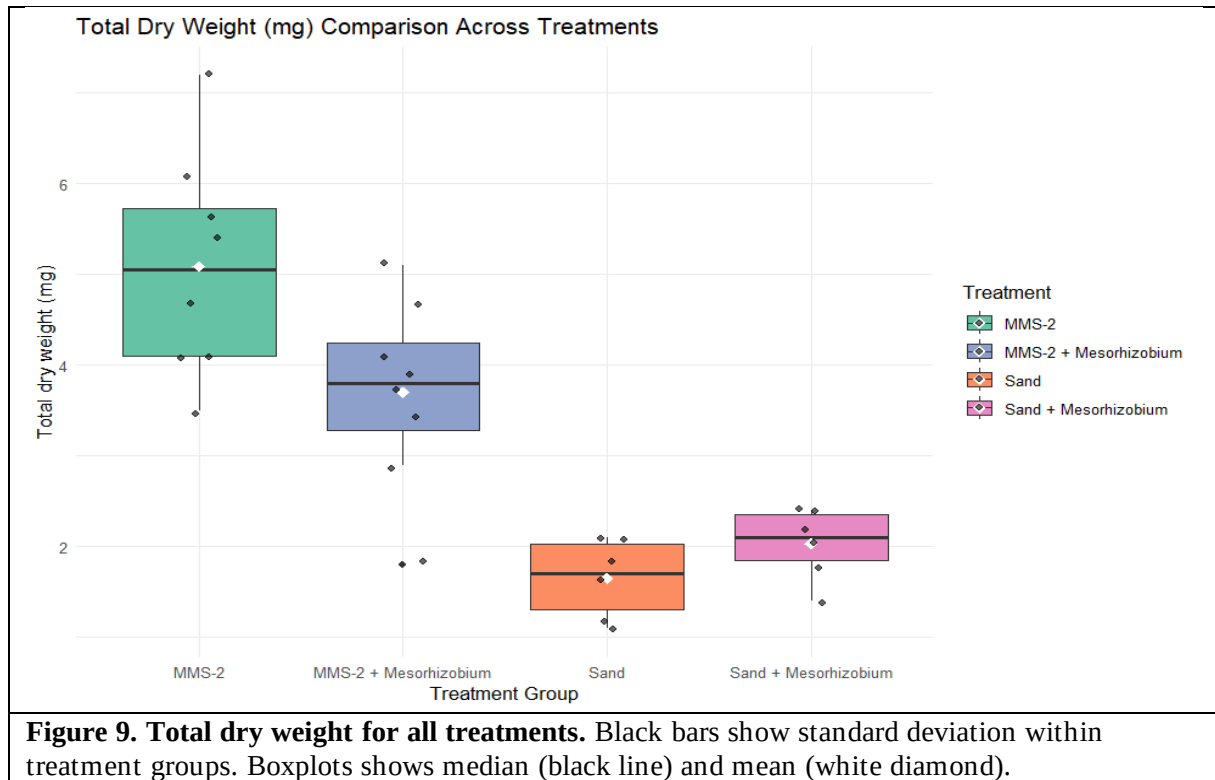
Figure 7. Nodules from *L. japonicus* roots. Left: Sand-grown plant with a single large, soft, yellow nodule. Right: MMS-2-grown plant with a smaller, hard, translucent nodule (reddish due to soil adhesion). Nodules were found only at the root base and no more than one nodule was found per plant.

The root fresh weight measurements revealed an unexpected pattern in sand-grown plants (figure 8). Despite their stunted growth and significantly shorter root systems, sand treatments displayed the highest root fresh weight (2.8 ± 0.9 and 2.6 ± 1.5 mg) to MMS-2 roots (2.0 ± 0.8 and 1.4 ± 0.4 mg). Statistical analysis revealed that there was a significant difference between soil type and root fresh weight (Dunn's test $p < 0.03$). However, this apparent parity disappeared when examining root dry weight, where MMS-2 roots retained 3-4 times more biomass after dehydration (1.1 ± 0.5 mg and 0.7 ± 0.3 mg for MMS-2 vs. 0.6 ± 0.2 mg and 0.5 ± 0.2 mg in sand). This translates to water content of 42.1%-49.1% for MMS-2 and 73.7%-78.7% for Sand treatments. All MMS-2 treatments displayed significantly higher total dry weight compared to sand treatments (figure 9) (Dunn's test, $p < 0.001$). All biomass parameters are displayed in table 3.

Treatment	Root fresh weight (mg)	Root dry weight (mg)	Shoot fresh weight (mg)	Shoot dry weight (mg)	Total dry weight (mg)
MMS-2	2.0 ± 0.8	1.1 ± 0.5	18.9 ± 4.2	4.0 ± 0.8	5.1 ± 1.2
MMS-2 + <i>Mesorhizobium</i>	1.4 ± 0.4	0.7 ± 0.3	16.5 ± 3.7	3.0 ± 0.8	3.7 ± 1.0
Sand	2.8 ± 0.9	0.6 ± 0.2	5.0 ± 1.7	1.1 ± 0.3	1.6 ± 0.4
Sand + <i>Mesorhizobium</i>	2.6 ± 1.5	0.5 ± 0.2	5.1 ± 1.6	1.5 ± 0.3	2.0 ± 0.4

Table 3. Mean $\pm$ standard deviation of root and shoot biomass parameters for *L. japonicus* grown under different treatments. Fresh and dry weights (in mg) are presented for roots, shoots, and total plant biomass. Plants were grown on either MMS-2 or nutrient-free quartz sand, with and without inoculation with *Mesorhizobium* sp. *LjRoot218*.





pH Dynamics of Mars Regolith Simulant

Initial characterization revealed MMS-2 to be highly alkaline, with a pH of 11.18 when suspended in water (1:2 w/v MMS-2:water). Attempts to lower the pH through titration with 2 M HCl demonstrated the soils strong buffering capacity. While incremental additions of HCl temporarily reduced pH to 5.85 (5 mL cumulative addition), the pH rebounded to 7.30 within one hour of venting and to a further 7.85 after 24 h (Figure 10).

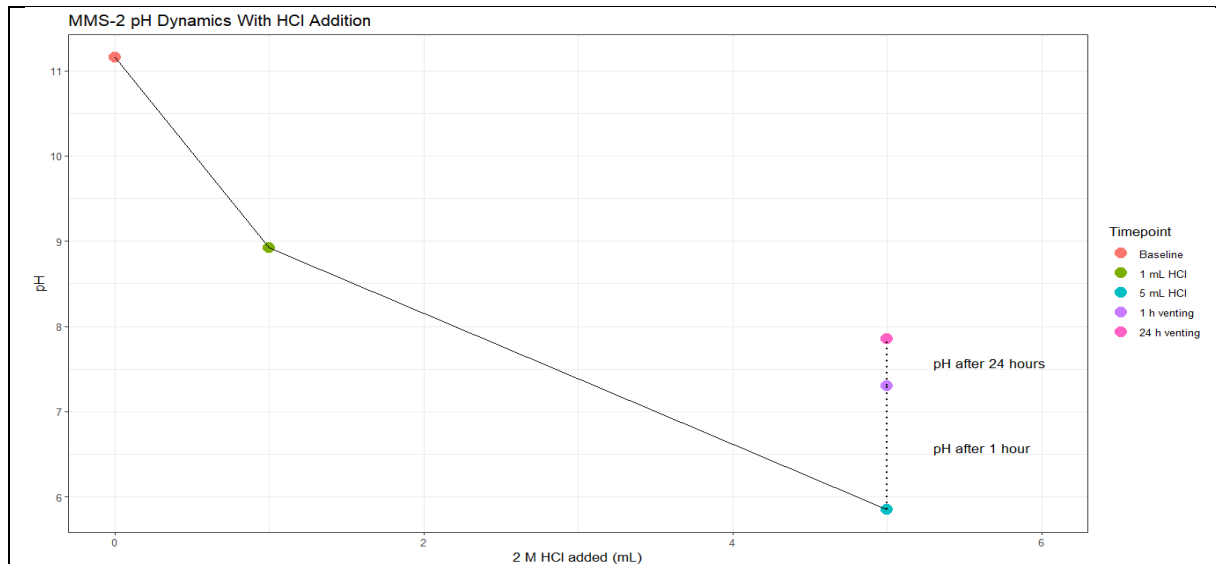


Figure 10. MMS-2 regolith pH dynamics in a 2:1 v/v water solution. At $t = 0$, 13.7 g MMS-2 was dissolved in 20 mL autoclaved tap water, shaken vigorously, and vented for 1.5 h. Graph shows initial regolith pH of 11.16. After 1 mL of 2 M HCl the pH was reduced to 8.92 and after an additional 4 mL (5 mL total) the pH further decreased to 5.85. After 1 hour of venting, the pH increased to 7.30 and after 24 h to 7.85 (dotted lines).

The buffering resilience was further evidenced by failed stabilization attempts with MES buffer (2:1 v/v, 0.1 and 0.2 M, pH 5.6). Immediate pH measurements after buffer application showed only a marginal reduction to 9.97, with values climbing back to 10.30 after 48 hours of venting. This resistance to external pH modification persisted across multiple buffer concentrations (0.1 and 0.2 M), with none achieving the target pH range for *L. japonicus* (6.4) in order for the adjusted soil pH treatment to be possible (figure 11).

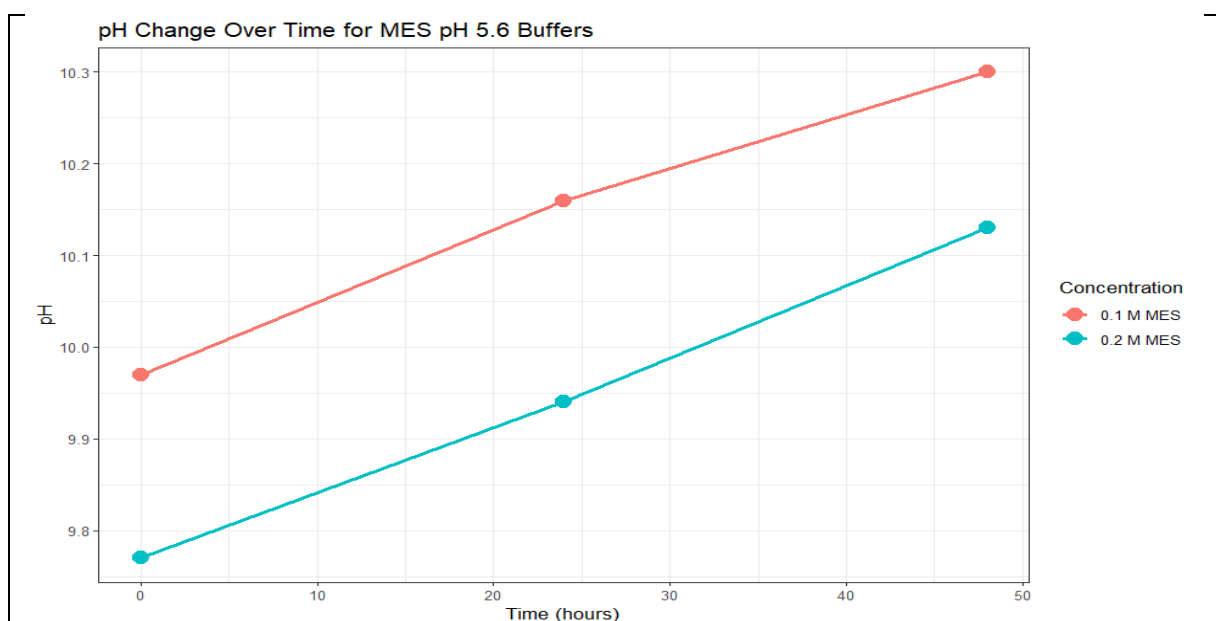


Figure 11. MMS-2 MES buffer solution pH change over time. The red line shows 0.1 M MES, and the blue line shows 0.2 M MES. Initial pH measured after 10 min, 24 h and 48 h of venting. pH of 0.1 M started at 9.97 then increased to 10.16 and 10.30. 0.2 M started at 9.77 and then increased to 9.94 and 10.13

The acid-base buffering characteristics of MMS-2 regolith were quantified through systematic titration of a 1:5 (w/v) soil-water suspension with 2 M HCl (0-6500 μ L) (figure 12). The suspension showed a linear progressive protonation behavior without a distinct sigmoidal transition from its initial alkaline conditions (pH 10.00) to strongly acidic (pH 1.37). At a pH of 1.90 the decrease slowed until it reached the pH of the HCl. This further indicates the incredibly strong buffering capacity of the regolith.

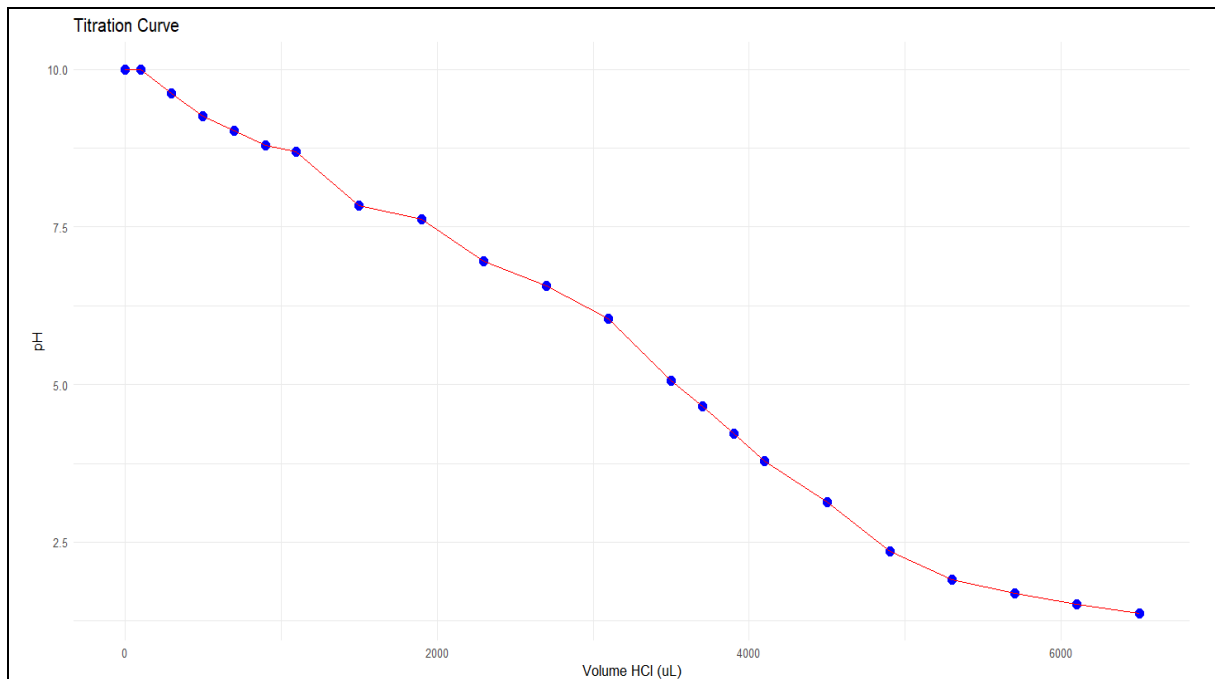


Figure 12. Titration curve of MMS-2 regolith suspension with 2 M HCl. A 1:5 (w/v) soil-water suspension was prepared by mixing 2 g of MMS-2 with 10 mL of autoclaved tap water and shaking for 1.5 hours. The suspension was titrated incrementally with 2 M HCl in 400 μ L steps, with pH measured after each addition. The curve shows a gradual decrease in pH from 10.0 to $\sim$ 1.5 with increasing acid volume, indicating the absence of a distinct equivalence point and highlighting the strong buffering capacity. The pH response was linear without the typical sigmoidal shape of weak acid-base systems.

Germination rate in Miller-Urey

After seven days of germination, visual analysis was performed on the germination rate of the seedlings. Every seed was examined based on if the outer shell of the seed was cracked and if parts of the plant protruded out. Seedlings on the positive control on Whatman paper that all successfully germinated, showed clear shoot elongation and cotyledon leaves (figure 13 top left). The seedlings on the MMS-2 showed a similar high germination rate with 15 of the 20 individuals successfully breaking the seed outer layer (Figure 13 top right). Out of these 14 even developed elongated shoots and cotyledon leaves, showing a success rate of 85% on simulated Martian soil under earth like conditions. More surprisingly, 5 of the 20 seeds in the Miller-Urey also broke the outer seed layer (figure 13 bottom left), with one seedling even developing a green hairy root. This 25% germination rate indicates that even in harsher

atmospheric conditions, a wet simulated Martian regolith still provides the conditions for *L. japonicus* to germinate.

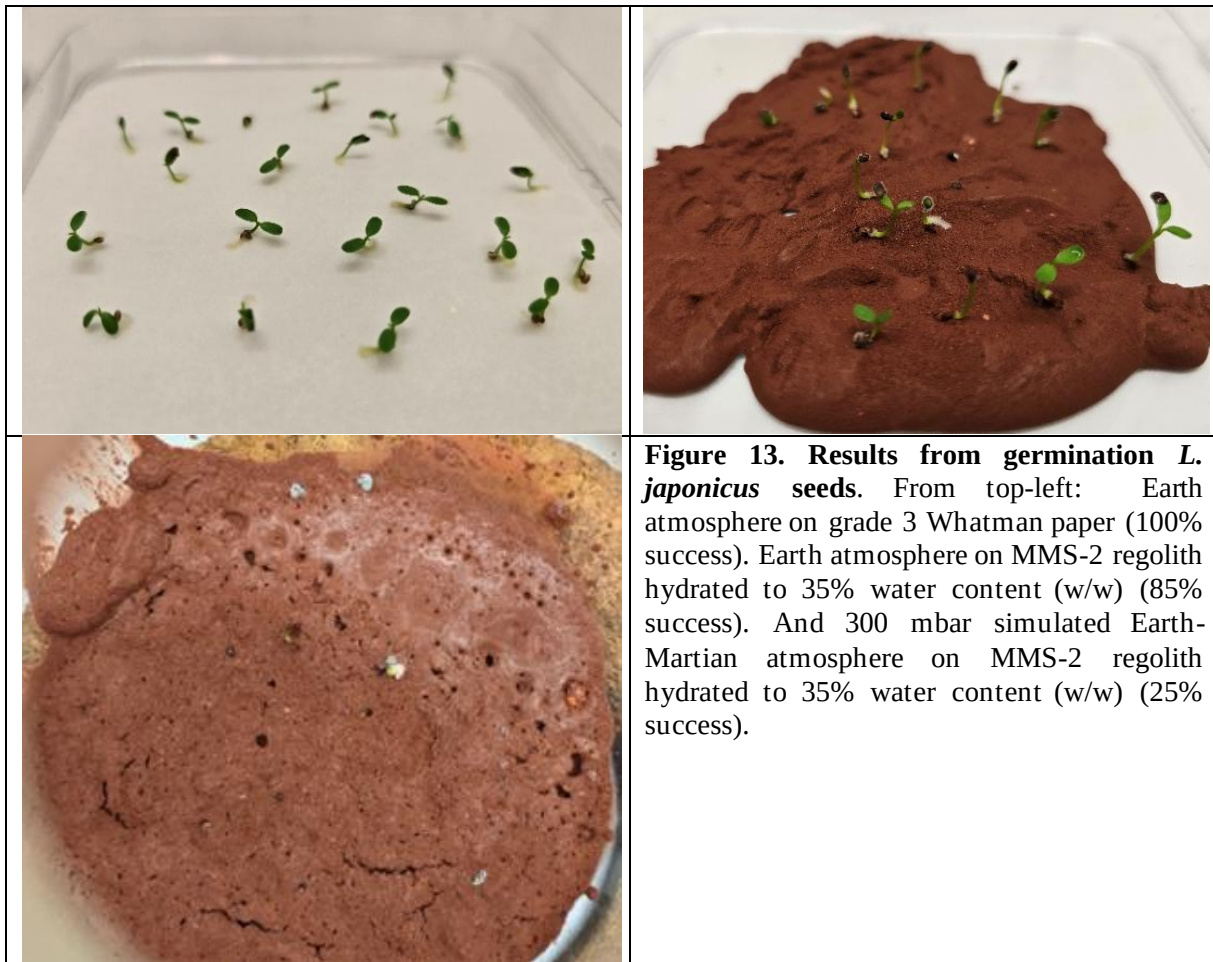


Figure 13. Results from germination *L. japonicus* seeds. From top-left: Earth atmosphere on grade 3 Whatman paper (100% success). Earth atmosphere on MMS-2 regolith hydrated to 35% water content (w/w) (85% success). And 300 mbar simulated Earth-Martian atmosphere on MMS-2 regolith hydrated to 35% water content (w/w) (25% success).

Discussion

Lotus japonicus grew significantly better on MMS-2 regolith simulant than on nutrient-free quartz sand, showing higher shoot and root development and dry biomass accumulation. Inoculation with *Mesorhizobium* sp. *LjRoot218* resulted in minimal nodulation but did not improve plant growth in either substrate. The highly alkaline pH of MMS-2 remained stable between pH 10–11 throughout the experiment, despite acid and buffer treatments. Seed germination was successful both under Earth atmospheric and Martian soil conditions, and under low-pressure Mars-like atmospheric conditions, though germination rates were lower at reduced pressure. These results demonstrate that *L. japonicus* can survive and grow under multiple Martian-like stressors.

Due to logistical constraints, all procedures outside the climate chamber were conducted in a shared laboratory (B3.13), which was simultaneously used for soil-related experiments. As a result, contamination from non-sterile soils may have occurred at undetermined stages. While the quartz sand was autoclaved prior to use to minimize microbial interference, the MMS-2

simulant was not sterilized. Owing to its fine, powdery nature, autoclaving would have introduced excessive moisture, rendering the substrate bulky, clumpy, and difficult to manage for planting. Moreover, given that MMS-2 is a synthetically produced regolith simulant, it was assumed to carry minimal biological contamination. Additionally, the climate chamber malfunctioned multiple times during the experiment, with humidity levels dropping below the target 55% for the first seven days and the climate chamber unexpectedly turning off for three days (day 25-28). This disruption coincided with increased leaf yellowing in some plants (Data repository “Plant_growthovertime_table”), suggesting stress from inconsistent environmental conditions.

Mesorhizobium inoculation did not significantly influence plant growth in either substrate. Root analysis revealed minimal nodule formation, and due to time constraints, further genotypic confirmation of nitrogen-fixing bacteria was not conducted. Thus, the potential benefits or drawbacks of inoculation remain unclear. In Rhizobium-legume symbiosis, the N₂ fixation process is strongly related to the physiological state of the host plant (Zahran. H., 1999). Therefore, the nitrogen fixating capabilities could be limited which could also have led to the yellowing in the leaves over the period as the lack of nitrogen leads to lower chlorophyll levels in plant leaves. Moreover, the low phosphorous levels in MMS-2 could have directly hindered this relationship as well as studies have shown that low phosphorus availability is known to directly hinder symbiotic relationships by impairing nodulation and nitrogen fixation (Martins et al., 2017). This review also points out salt stress as a similar limiting factor for symbiosis. Ramírez et al. (2019) found extreme salt stress indicated by average maximum stomatal conductance <50 mmol H₂O m⁻² s⁻¹ in plants grown on Martian simulation soil. This research has not used salt stress as a limiting factor on Mars and future experiments should look further into the effect of Rhizobium-Legume relationships under salt stress in Martian soil.

Interestingly, Rainwater and Mukherjee (2021) reported successful *Mesorhizobium* inoculation and increased plant biomass in legumes grown on MMS-2, alongside strong nifH (a reporter gene for nitrogen fixation) expression in the root nodules, indicating effective symbiosis. This difference may be explained by the differences in bacterial strains *Sinorhizobium* vs. *Mesorhizobium* or inoculation density (OD 0.8 vs. 0.2 in this study). This trade-off was made in consideration to not succumb the plants to stress from a high bacterial count on nutrient-poor soil. The original LjR218 strain consisted of a few large bacterial colonies, which made inoculation of a distinct genotype difficult. It was decided to use the big colony anyway due to time constraints and because the exact genotype was irrelevant to the experiment as long as a nitrogen-fixating bacterium was present.

Surprisingly, Rainwater and Mukherjee (2021) also found the most successful plant growth in the sand treatment, with greater biomass compared to other substrates. This in contrast to my findings, where plant growth in sand was significantly lower compared to other conditions (Tukey HSD $p < 0.001$). In their experiment they supplemented seeds with 1 mM Gibberellic acid (GA3) medium after sterilization. Other experiments similarly claim GA3 enhances plant

growth, leaf and branch count, and the fresh weight (El-Tohmamy et al., 2023) on plants cultivated on sand. This could indicate that plant hormones like GA3 are crucial for growth on these types of soil both on earth and Mars. However, this does not explain the significant increase in plant growth under MMS-2 compared to Sand in my experiment and suggests that *L. japonicus* can access nutrients within the regolith despite its high metal oxide content.

Most of the metal-oxides in MMS-2 are water insoluble and are therefore not readily taken up by the plant's root system to function as nutrients. However, previous research has shown that some metal oxides like hematite (Fe_2O_3), which makes up about 18% of the Martian surface, can undergo surface hydroxylation, forming hydrophilic $-\text{OH}$ groups even at low humidity levels. As humidity increases, molecular water adsorbs onto the surface, forming a monolayer (Yamamoto et al., 2010). This hydration shell supports proton and ion exchange, making the hematite surface chemically active and capable of enhancing nutrient solubilization and interaction with root exudates. Such water retention and reactivity may be particularly advantageous in dry, nutrient-poor substrates like MMS-2.

Studies on metal oxide nanoparticles (NPs) further support their role in plant growth. For example, hematite NPs increased legume biomass by up to 830% (Boutchuen et al., 2019), likely through surface-mediated metabolic effects rather than direct nutrient uptake. Similarly, magnesium oxide NPs improved chlorophyll synthesis and biomass in mustard (Gautam et al., 2023). However, the high iron and magnesium concentrations in Martian regolith ($\sim 160\times$ and $\sim 50\times$ higher, respectively, than NP doses used in these studies) could induce nutrient imbalances or oxidative stress (Harish et al., 2023).

Conversely, aluminum oxide NPs have been shown to cause cellular damage and inhibit root elongation in wheats (Yanık, F & Vardar, F. 2015). Similarly high Aluminum concentration in alkaline soils can restrict root growth, microbiological activities, and the uptake of essential elements like K, Ca, and Mg (Shetty et al., 2021). Future experiments should investigate the effects of controlled metal oxide additions to sand on *L. japonicus* growth.

Interestingly, the plants grown on sand showed the highest mean root fresh weight compared to the MMS-2 treatments, while having significantly smaller root systems. This may be explained by the fact that unlike MMS-2, the quartz sand was nutrient poor. As such, nutrient availability was unlikely to drive root expansion or biomass accumulation. However, sand's coarse texture and low water-holding capacity may have introduced early-stage water stress. In response, plants may have developed thicker, denser roots to enhance water uptake efficiency under lower hydration. Additionally, the higher fresh weight likely reflects increased water content within root tissues rather than structural biomass, as suggested by the significantly lower dry weight measured in sand-grown plants. This contrast between fresh and dry weight implies that root water retention—not nutrient acquisition—was the primary factor influencing early root morphology in the sand treatment.

A secondary experiment assessed seed germination in MMS-2 and a simulated Martian atmosphere. Before the initial experiment with sterilized seeds, the experiment was performed with unsterilized seeds under identical conditions. Surprisingly, the seeds on Whatman paper were the only seeds not germinating. It was hypothesized that the unsterilized seed coats were too thick to absorb the moisture on the Whatman paper. The experiment was then repeated with sterilized seeds while subsequently an experiment was performed using non-sterilized seeds in a petri-dish with three layers of Whatman paper all soaked in water. Crevices were formed in the paper for the seeds to lay in. After four days the unsterilized seeds all germinated proving my hypothesis was correct.

Unexpectedly, some seeds germinated in MMS-2 with an altered atmosphere. The atmospheric conditions were predetermined based on available tools and time in the lab. First, the Miller-Urey was not precise enough to create Martian atmospheric gas proportions under such low pressure. Considering it was expected that the seeds would not germinate under these pressure levels at all, it was therefore decided to form a pressure of ~300 mbar, which resembles the pressure levels at Mount Everest and therefore the theoretical limit of plant growth on earth. However, this meant that any gas ratio would become arbitrary compared to the atmosphere on Mars, considering the pressure and therefore available gas for the seeds would be up ten-fold. Secondly, there was no oxygen tank available in the lab, meaning pressurized air had to be used as O₂ source resulting in imprecise N₂ and CO₂ levels. Even though all this limited extrapolation to the Martian environment, these results highlight the resilience of *L. japonicus* under extreme conditions.

MMS-2 exhibited remarkable buffering capacity, resisting acidification despite repeated additions of 2 M HCl. Initial pH measurements of ~10–11 exceeded literature values for Martian regolith (pH ~8) (Wamelink & Pouwels, 2024; Sutter, 2021). Multiple acid and buffer treatments, including MES buffer, resulted only in marginal and temporary pH reductions. As plant pH treatments became unfeasible, the focus shifted toward understanding the underlying buffering behavior. An acid-base titration revealed a linear response without the sharp equivalence point typical of classic titration curves, indicating continuous proton neutralization over a wide pH range. This strong buffering is likely driven by the high oxide content of MMS-2. Hematite (Fe₂O₃) for instance, forms hydration shells that support proton and ion exchange, while CaO, MgO, and Na₂O readily generate hydroxide ions (e.g., Ca(OH)₂, Mg(OH)₂, NaOH), maintaining alkalinity and causing the pH to rapidly rebound after acid addition. Although SO₃ could theoretically hydrolyze into sulfuric acid (H₂SO₄), sulfur in Martian regolith primarily exists as stable sulfates (e.g., CaSO₄, MgSO₄, FeSO₄) (Franz et al., 2019; Bishop et al., 2014), which are neutral or only weakly acidic in solution, contributing little to buffering. Despite these explanations, the observed starting pH (10–11) remains surprisingly high, with no comparable values previously reported for MMS-2. This discrepancy suggests that the buffering behavior of Martian simulants may not yet be fully understood. Future research could dissect the specific contributions of individual oxides by isolating and rehydrating them

separately, while advanced mineralogical techniques such as X-ray diffraction (XRD) or scanning electron microscopy with energy-dispersive spectroscopy (SEM-EDS) could further clarify the structural components driving the strong alkaline buffering in MMS-2.

In summary, this study investigated whether *Lotus japonicus* can survive and grow using resources available on Mars, while facing environmental stressors such as nutrient limitations, extreme alkalinity, and an altered atmosphere during seed germination. The findings demonstrate that *L. japonicus* is capable of germination, biomass accumulation, and root development on Martian soil simulant MMS-2 despite its nutrient-poor and highly alkaline nature. While inoculation with *Mesorhizobium* resulted in limited nodulation and no significant growth benefit, the species still tolerated nitrogen absence remarkably well. Seed germination under reduced-pressure and Mars-like atmospheric conditions further confirmed the resilience of early plant development stages. Although challenges remain like the presence of toxic chemicals like perchlorates and the high soil pH, these results highlight the potential of legumes like *L. japonicus* for future plant-based life support systems on Mars.

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Data repository

All data, analysis scripts, and supplementary materials related to this thesis are available in a dedicated GitHub repository:

https://github.com/MielHout/BScProject_HoutenbosMiel_PlantGrowthOnMars_DataRepository.git

The repository includes:

- Raw and processed data files for all three experiments
- R scripts used for data cleaning, analysis, and figure generation
- Photographic records documenting *L. japonicus* growth over time
- Soil titration and germination measurements
- Experimental methods, protocols, and data management documentation

This repository ensures transparency, reproducibility, and accessibility for future research on plant growth under simulated Martian conditions.

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Appendix

Protocols

Sterilization and germination of *L. japonicus* seeds

1. Scarify seeds with sandpaper (P120): use a small mortar and a small piece of sandpaper (1.5 x 1.5 cm) and rub the seeds with your thumb, applying medium force. The seeds are sufficiently scarified when they look almost shiny.
2. Prepare 10 mL sterile water with 1 mL of sodium hypochlorite (bleach from Jumbo supermarket, <2.5% hypochlorite) in a 15-mL Falcon tube, add the seeds, and shake for 20 min at 185 rpm or incubate on a rotary shaker.
3. Wash 5x with sterile water on the sterile bench, pouring the liquid carefully out of the tube.
4. With sterile forceps, place a sterile (autoclaved) Whatman paper (~11.5x11.5 cm) in a 12x12 cm squared Petri dish; add sterile water to wet the paper; there should be a little bit (~3 mL) excessive water that will be soaked up over time.
5. Pour sterilized seeds onto the paper, using what is left of the rinsing water in the 15-mL tube; spread the seeds with sterile forceps; make sure the seeds do not sit too close to each other.
6. Close the plate and seal with Parafilm, place plates in room with plenty sunlight
7. Germinate for 3 days.

TY growth medium for *Mesorhizobium*

Concentrations for 1 L TY medium

Tryptone	5 g
Yeast extract	3 g

For solid medium:

Bacto Agar	15 g (1.5% w/v)
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After autoclave:

1M sterile CaCl ₂	10 mL (1% v/v)
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Radiant Streaking Procedure

1. Sterilize an inoculation loop by keeping it in a flame until it is red hot and allow it to cool.
2. Gently scoop a bacterial colony with the inoculation loop.
3. Lift the Petri plate you want to inoculate in your left hand and hold it at a slight angle.
4. Spread the inoculum over the near edge of the agar plate using a gentle zigzag motion.
5. Sterilize the loop and cool it by pressing it gently in the agar. Streak from the point of primary spread in a radial direction up to the far edge of the Petri plate. (Be sure to streak the lines far away from each other.)

6. Re-flame the loop and allow it to cool. Then draw horizontal lines crossing the radial streaks.
7. Repeat this process three times.
8. The fourth time dilute the streak, so the zigzag line does not cross itself.

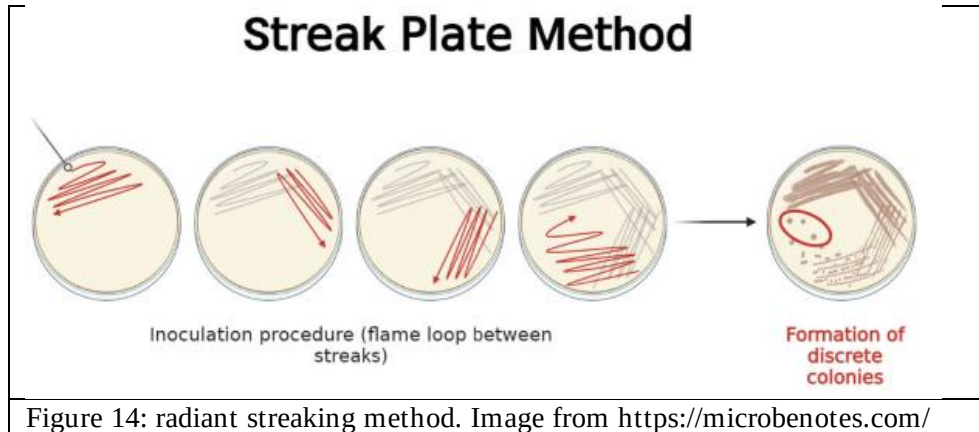


Figure 14: radiant streaking method. Image from <https://microbenotes.com/>

Bacterial inoculation

1. Start a liquid culture of the strain (e.g., LjRoot218) in 3 mL TY+CaCl₂ medium.
2. Incubate shaking at 25°C for 3 days.
3. Harvest by spinning the culture at 3500 rpm for 5 minutes.
4. Carefully discard the supernatant.
5. Wash the pellet in 3 mL 10 mM MgSO₄.
6. Measure the OD₆₀₀ of the culture.
7. Make a dilution of the culture with 10 mM MgSO₄ to a final OD of 0.2.
8. Open the agar plates with the plants on the sterile bench.
9. Distribute 500 µL of the diluted bacterial culture horizontally across the root tips.