



RESEARCH ARTICLE

Lunar regolith simulant for cultivation of plants: seed germination and early growth study of plant vitality

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Abstract

Bioregenerative life support systems (BLSS) designed to produce food crops in future crewed missions to the Moon or Mars consider *in-situ* resource utilisation (ISRU). Lunar regolith is, therefore, in focus for future technologies of farming on lunar bases. We tested germination and early growth of broccoli plants (*Brassica oleracea* var. *botrytis italica*) in Murashige-Skoog liquid medium with addition of leachate from a lunar regolith simulant. As the additions to growth, differently diluted water and acidic leachates were used. Physiological status of the germinating plants was evaluated by chlorophyll fluorescence parameters related to plant vitality (relative fluorescence decline – Rfd) and photosynthetic performance of photosystem II (1) potential (F_V/F_M) and (2) effective quantum yield of PSII (Φ_{PSII}), photochemical quenching referring to number of open PSII reaction centres. Both water and acidic leachates inhibited plant growth, however, the extent of growth limitation was dilution-dependent. Full inhibition of germination was apparent when undiluted acidic leachate was added. However, 50% dilution (and higher) resulted in seed germination and the early growth. No negative effects of the water dilutions on F_V/F_M as well as Φ_{PSII} , were apparent in 15 days old plants, their cotyledonary and the first primary leaves, in particular. Similarly, qP and Rfd showed no sign of either water or acidic leachate addition effect. Although photosystem II-related parameters exhibited no negative effect of the leachates addition, a growth of plants was found dilution-dependent: higher degree of dilution resulted in a more pronounced reduction in plant projection area. In spite of the growth rate reduction (compared to untreated control), properly diluted water and acidic leachates from lunar regolith and/or its simulants might be used in follow up studies focused on plant species prospective for future cultivation in Moon-based stations with temporary or permanent crew.

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Introduction

Considering a lunar base construction, its running and effective performance, cultivation of plants in such a base is a key component because of (1) CO₂ fixation resulting in biomass production to be utilised as food source for a base crew, (2) oxygen production and functioning as (3) biological life supporting systems (BLSS). The first short-term lunar test of BLSS was done just recently using the plants cultivated on lunar surface regolith (Xie *et al.*, 2024). The lunar experiment lasted 15 days and focused on germination rate and initial plant growth under intense radiation, low gravity and prolonged intense light. A substrate used in this study was vermiculite – a silicate mineral with chemical formula (Mg, Fe⁺⁺, Al)₃(Al, Si)₄O₁₀(OH)2.4(H₂O) combined with a nutrient solution. However, for future lunar experiments, as well as plant cultivation in lunar base facilities, lunar regolith should be used in order to promote in-situ resource utilisation (ISRU) which make a base maintenance, lunar research and exploration and general crew activities less costly and more effective. Recently, an effort aimed to ISRU led to a comprehensive review of the ways to improve lunar regolith for successful plant cultivation (Ding *et al.*, 2024).

To use lunar regolith as a substrate for plant cultivation during the first missions to the Moon has been suggested in the studies focusing on the way of living of future crews inhabiting a lunar base (Lytvynenko *et al.*, 2006). Study of Paul *et al.* (2022) that used regolith samples from Apollo 11, 12 and 17 showed that *Arabidopsis thaliana* germinated and grew in these lunar regoliths. The study identified several problems, such as slow growth rate, malformations in plant morphology and molecular biology markers. Moreover, transcriptomic study was done as a part of the experiments in the regolith-germinating plants that identified differently-expressed genes associated with salt – and metal-induced stress resulting in reactive oxygen species (ROS) formation. Therefore, the study of Paul *et al.* (2022) stimulated follow up studies using lunar regoliths’ simulants. The studies focused on several aspects of biofertilisation of Lunar and/or Martian regolith (Duri *et al.*, 2022) or their simulants (Zhao *et al.*, 2024) with organic matter. Biofertilisation represents a prospective way to increase the agricultural potential of these substrates. Such systems utilising regolith and regolith simulants might be used in future space farming (Caporale *et al.*, 2023). Moreover, the number of plant species tested in such growth systems has been increasing in the last decade (e.g., Eichler *et al.*, 2020; Fackrell *et al.*, 2020; Gonçalves *et al.*, 2024; Rickard *et al.*, 2021).

Lunar regolith simulant overviewed recently by Zhang *et al.* (2021) is a prospective alternative for the studies focused on plant growth. The cultivation in lunar simulants, however, brings some specific problems related to the fact that lunar regoliths and/or their simulant differ in chemical composition and nutrients bioavailability from natural Earth-based soils. In general, regolith simulants are generally extremely poor in both nutrients and organic matter needed for successful plant growth (Seiferlin *et al.*, 2009). Therefore, especially under such extreme conditions and in the absence of external nutrient inputs, organic amendment is particularly important. Addition of organic matter is one of strategies applied in lunar (see e.g., Caporale *et al.*, 2022) or Martian regolith simulant, as shown e.g., by (Zhao *et al.*, 2025 for *Arabidopsis. thaliana*). Recently, the role of bacteria on the improvement of lunar regolith (simulant properties) have been studied. For example, the effect of phosphorus-solubilizing *Bacillus mucilaginosus*, *Bacillus megaterium* and *Pseudomonas fluorescens* on an increase in phosphorus bioavailability for plant growth was reported by Xia *et al.* (2023). Such effect is possible due to by dissociating insoluble phosphorus from the lunar regolith. Apart from the improvement of lunar regolith by microbial environment modification, several other approaches have been tested

involving *e.g.* addition of organic waste with earthworms (Wang *et al.*, 2025), addition of antioxidants into the regolith (Barcenilla *et al.*, 2024).

Recently, a great number of germination experiments have tested plant capability to germinate and perform satisfactory growth in lunar regolith simulants. Wamelink *et al.* (2014) tested germination and early plant growth (50 d) of 14 different species in lunar and Mars soil simulants and found satisfactory germination rate. Although several biometrical parameters, as well as biochemical and molecular biology markers have been monitored during the early growth, less attention has been paid to the evaluation of photosynthetic processes in plants cultivated in lunar regolith and/or analogues.

Heavy metals, like aluminium and chromium are also present in the extra-terrestrial regoliths. Aluminium is known to disturb plant growth (Kocjan *et al.*, 2025) and even lead to cell/plant death caused by Al-induced oxidative stress (Chakraborty *et al.*, 2024). Previous study (Coufalík *et al.*, 2024) determined the mobile fraction of metals in lunar regolith simulant which was selected as model substrate for testing plant germination and early growth. Therefore, the aim of this study was to evaluate germination of *Brassica oleracea* var. *botrytis italica* in leachates from lunar regolith simulant. The leachates were extracts from the lunar regolith simulant (National Technical University of Athens) extracted either by acetic acid or deionized water, containing Cd, Cr, Zn, Ni, Pb and Cu (for details see Coufalík *et al.*, 2024). In our experiment, we tested hypothesis that water (aqueous) leachates would provide a better conditions for seed germination than acetic ones due to lower release of metal ions from the regolith particles. Since the release of the above-mentioned heavy metal ions is medium-dependent (different content of salts and acids), we used two contrasting options for dilutions, *i.e.* water (as a general reference) and acid (as most extreme).

In this study, we focused on a Lunar regolith simulant (Argyrou *et al.*, 2024; Coufalík *et al.*, 2024; Stavrakakis *et al.*, 2022) developed by the experts from the National Technical University of Athens (NTUA), Greece (*see* also Material and Methods) which showed high degree of similarity to lunar regolith and was, therefore, proposed as a substrate for plant cultivation in space analogue habitat greenhouses. The study aimed to germinate and grow broccoli (*Brassica oleracea* var. *botrytis italica*) in Murashige-Skoog (MS) liquid medium with addition of aqueous and/or acidic leachate. We hypothesised that experimental plant growth and primary photosynthetic processes will be negatively affected by the leachates. However, a degree of leachate dilution done before the addition to the MS medium would alleviate negative effects and stimulate plant growth and development in comparison to control (pure MS medium). Therefore, we decided to monitor several photosynthetic parameters, particularly those related to evaluation of photosystem II, during a germination study in order to point out crucial point in optimising dilution of leachates from lunar regolith simulants (in this study) and watering and/or nutrient solution composition to optimise growth in regoliths in general (a follow up study mimicking a lunar greenhouse set-up).

Material and methods

Lunar regoliths characteristics

The lunar regolith simulant utilised in our study was developed at the National Technical University of Athens (NTUA), Greece, and is a part of the fifth batch (L5) produced by the team. The lunar regolith simulant was developed based on the Apollo 15 samples (Sample No. 15260 – Duncan *et al.*, 1975). Multi mineral-rock approach during the simulant development process was used (Argyrou *et al.*, 2024; Stavrakakis *et al.*, 2022). The simulant evaluation has proven that it highly resembles the lunar surface (Coufalík *et al.*, 2024). The similarity was in mineralogy, grain size and chemical match, which was also evaluated based on the method of Schrader *et al.* (2009). The method showed a 95.83 chemical similarity (Figure of Merit, FOM) to the lunar regolith. Detailed chemistry of the simulant and its correlation with the Lunar surface data of the Sample No 15260 can be found in Table 1.

Table 1. Chemical composition of L5 (Lunar regolith simulant) compared to the Apollo 15260 sample analysis (Duncan *et al.*, 1975) adopted from Coufalík *et al.* (2024)

	Na ₂ O	MgO	Al ₂ O ₃	SiO ₂	P ₂ O ₅	SO ₃	Cl
Duncan 75	0.39	10.77	16.49	46.61	0.22	0.08	0.00
L5 Measured	2.51	10.95	16.12	44.92	0.25	0.39	0.00
	K₂O	CaO	TiO₂	Cr₂O₃	MnO	FeO (Fe₂O₃)*	SUM
Duncan 75	0.19	11.21	1.51	0.00	0.16	12.35	100.00
L5 Measured	0.38	9.83	0.88	0.29	0.07	13.41	100.00

Note: *All iron measured as FeO.

Table 2. Extractable fractions (%) of metals in deionised water and 0.11 mol/L acetic acid in the leachates from lunar regolith simulant prepared by the National Technical University of Athens, Greece

	Lunar regolith	
	Deionised water	Acetic acid (0.11 M)
Cd	0.00	34.03
Cr	0.05	3.49
Cu	0.26	2.88
Ni	0.07	4.77
Pd	0.50	1.17
Zn	0.00	54.76

Lunar regolith simulant leachate

Leachates were prepared from the lunar regolith analogue using deionised water and 0.11 M acetic acid. The extraction of a leachate was done on a shaker (GFL 3018, Germany) in 0.11 mol/L CH₃COOH (Suprapur, 96%, Merck, Germany) in a ratio of 0.5 g of sample per 20 mL of solution at laboratory temperature for 16 h. Extraction in deionised water was carried out under the same conditions. Then, the extracts were centrifuged (Centrifuge 5804R, Eppendorf AG, Germany) at 9000 g for 10 min, and collected into HDPE scintillation vials (Kartell, Italy).

Plant cultivation

Individuals of broccoli (*Brassica oleracea* var. *botrytis italica*) were cultivated from seed in a Murashige-Skoog (MS) liquid medium (for medium composition, see Duchefa Biochemie, M0231.0050). The seeds were put into individual holes of microbiological plate, supplied with particularly diluted leachates (see Table 3) combined with a powdered MS medium (for medium specification, see Table 6). The seeds were provided equal volume of medium (1.0 ml per hole) at the beginning of germination. Control treatment was represented by exclusive MS medium. During germination and the early growth period, the microbiological plates were covered by a 12.6 × 8.2 × 11.2 cm (l × w × h) transparent minibox and sealed by a Teflon (PTFE) lab tape around the margins, to prevent evaporation of the medium. Then, cultivation was carried out in a growth chamber (*T* = 21°C, PAR = 120 μmol m⁻² s⁻¹) for 14 d. During cultivation, germinating plants took medium in a slow rate reducing its volume in a hole. Therefore, due to uptake of medium and variety of directions of initial root growth including the root growth above water/medium upper surface, the germinating roots did not grow under permanent immersion. However, the medium was refilled regularly (typically once a week) to maintain volume of 1.0 ml to provide sufficient amount for successful plant germination and growth.

Table 3. Abbreviations and explanations of the treatments used in the germination experiment of *B. oleracea*. MS – Murashige-Skoog medium

Abbreviation	Treatment	Note
Control	Control, exclusively MS medium, no addition	Seeds germinated in 1.0 ml of the MS in a hole
W10D	10 times diluted water leachate	Powdered MS medium (concentration 4.4 g L ⁻¹) was added into the W10D leachate forming a volume of 1.0 ml filled into a hole
W100D	100 times diluted water leachate added to MS	Powdered MS medium (concentration 4.4 g L ⁻¹) was added into the W100D leachate forming a volume of 1.0 ml filled into a hole
A10D	10 times diluted acidic leachate added	Powdered MS medium (concentration 4.4 g L ⁻¹) was added into the A10D leachate forming a volume of 1.0 ml filled into a hole
A25D	10 times diluted acidic leachate added	Powdered MS medium (concentration 4.4 g L ⁻¹) was added into the A25D leachate forming a volume of 1.0 ml filled into a hole
A50D	10 times diluted acidic leachate added	Powdered MS medium (concentration 4.4 g L ⁻¹) was added into the A50D leachate forming a volume of 1.0 ml filled into a hole
A75D	10 times diluted acidic leachate added	Powdered MS medium (concentration 4.4 g L ⁻¹) was added into the A75D leachate forming a volume of 1.0 ml filled into a hole
A100D	10 times diluted acidic leachate added	Powdered MS medium (concentration 4.4 g L ⁻¹) was added into the A100D leachate forming a volume of 1.0 ml filled into a hole

Plant growth and primary photosynthesis

The effects of acidic and water leachate on *B. oleracea* growth and primary photosynthetic performance were monitored during 2 weeks after germination. Functioning of photosystem II of the emerging plants was evaluated by chlorophyll fluorescence imaging system (Open FluorCam fluorometer, Photon Systems Instruments, Drásov, Czech Republic) by slow Kautsky kinetics supplemented with quenching analysis using the method described earlier (Barták *et al.*, 2021). Before experiments, *B. oleracea* plants were dark-adapted for 15 min (the time was tested before the experiment and found sufficiently long to open photosynthetic PSII centres). A single measurement started with an exposition of the experimental plants to low light for 30 s in order to induce the basal chlorophyll fluorescence F_0 , which is emitted from chlorophyll molecules located in light harvesting complexes. In this state, reaction centres of PSII (RC PSII) remained open. Then, a 2s-lasting saturation pulse was applied to close the RCs PSII and, therefore, induce the maximum chlorophyll fluorescence level (F_M). After the saturation pulse, a 30 s dark period followed to allow dark relaxation of chlorophyll fluorescence signal. After this period, the plants were exposed to a 300s-lasting actinic light (AL) period. During the light period, polyphasic rise and decline of chlorophyll fluorescence (slow Kautsky kinetics) was monitored. First, a rapid increase to a peak value (F_P) was achieved followed by a slow polyphasic decline in chlorophyll fluorescence to a steady-state chlorophyll fluorescence (F_S). The F_S level was measured at the end of the AL period. After another saturation pulse applied at the end of AL period, maximum chlorophyll fluorescence in the light-adapted state (F_M') was evaluated). Whenever the AL was switched off, the background chlorophyll fluorescence (F_o') was recorded. A final saturation pulse was given then to induce F_M'' level after a 40 sec dark period. From the above specified chlorophyll fluorescence data, the chlorophyll fluorescence parameters (for definitions see Roháček, 2002) were finally calculated using the formulae specified in Table 4.

Table 4. Chlorophyll fluorescence parameters used in this study. For more details see Roháček (2002)

Abbreviation	Description	Equation
F_v/F_M	Potential quantum yield of photosystem II	$F_v/F_M = (F_M - F_0)/F_M$
Φ_{PSII}	Effective quantum yield of PSII	$\Phi_{PSII} = (F_M' - F_S)/F_S$
qP	Photochemical quenching (Fraction of PSII centres in open states)	$qP = (F_M' - F_S)/(F_M' - F_0')$
Rfd	Relative decrease of chlorophyll fluorescence (Vitality ratio – Lichtenthaler <i>et al.</i> (2005))	$Rfd = (F_P - F_S)/F_S$

Results

Germination and growth rate

Water leachates when added to a MS medium in the two experimental dilutions (W10D, and W100D treatments) did not cause full inhibition of seed germination as recorded for some acidic leachate treatments = Figure 1 (*see below*). The early growth of *B. oleracea* was found dilution-dependent, lower growth rate was recorded for W100D than W10D treatment the rate of growth, however, was found lower for the W10D and W100D treatments than for a MS medium.

Acidic leachate prepared from the lunar regolith simulant caused 100% suppression of *B. oleracea* germination (Figure 2) due to toxic elements effects that limited germination (for the underlying physiological mechanisms, *see Discussion*). Diluted leachates added to the MS growth medium showed concentration-dependent response. While the suppression of germination was still apparent in the little diluted leachates (*i.e.* A10D, A25D treatments), the greater dilution treatments (A50D, A75D and A100D) led to seed germination. The early growth showed fastest rate and most pronounced development of cotyledons area in control (*i.e.* plants germinated exclusively in a MS medium – *see pixel area growth in Figure 3*). Germination and growth in the acidic leachate treatments showed an increase in time. However, the response was dilution-dependent. Higher dilution of acidic leachates led to a decrease in growth. The final pixel area ‘occupied’ by cotyledons, hypo- and epicotyl, recorded on the last day of experiment (day 18), decreased with the dilution as follows (related to control = 100%): A50D (96.6%), A75D (55.5%) and A100D (28.2%).

Primary photosynthetic processes

The effects of water and acidic leachates on the physiological processes in *Brassica oleracea* var. *botrytis italica* related to primary photosynthesis were monitored by several chlorophyll fluorescence parameters (F_v/F_M , Φ_{PSII} , qP and Rfd – for definition and meaning, *see Table 4*). Since all of them are associated with photosystem II and considered indicators of PSII functionality, the cultivation time dependent decrease in their values (Figures 5 and 6) is associated with gradual limitation of primary photosynthetic processes in PSII.

Capacity of photosynthetic processes in PSII (F_v/F_M) declined with the time of cultivation in W10D, and W100D treatments (Figure 5), however, the decline was similar to F_v/F_M time course in control plants. Moreover, no negative effect of the water dilutions on F_v/F_M were apparent, in spite of the fact that the final F_v/F_M value (day 15 in Figure 5a) were slightly higher (statistically insignificant) for W10D than W100D. There was no effect of acidic leachates on F_v/F_M since the time courses of F_v/F_M recovered for A50D, A75D and A100D treatments were comparable to untreated control (MS solution) – *see Figure 6a*. Similarly, to water leachate, A100D treatment led to a slight decrease in F_v/F_M value (compared to control) at the end of cultivation time.

Effective quantum yield of photochemical photosynthetic processes in PSII (Φ_{PSII}) is considered an indicator of photosynthetic linear electron transport in between PSII and PSI as well as overall photosynthesis expressing an equilibrium between primary photochemical and secondary biochemical

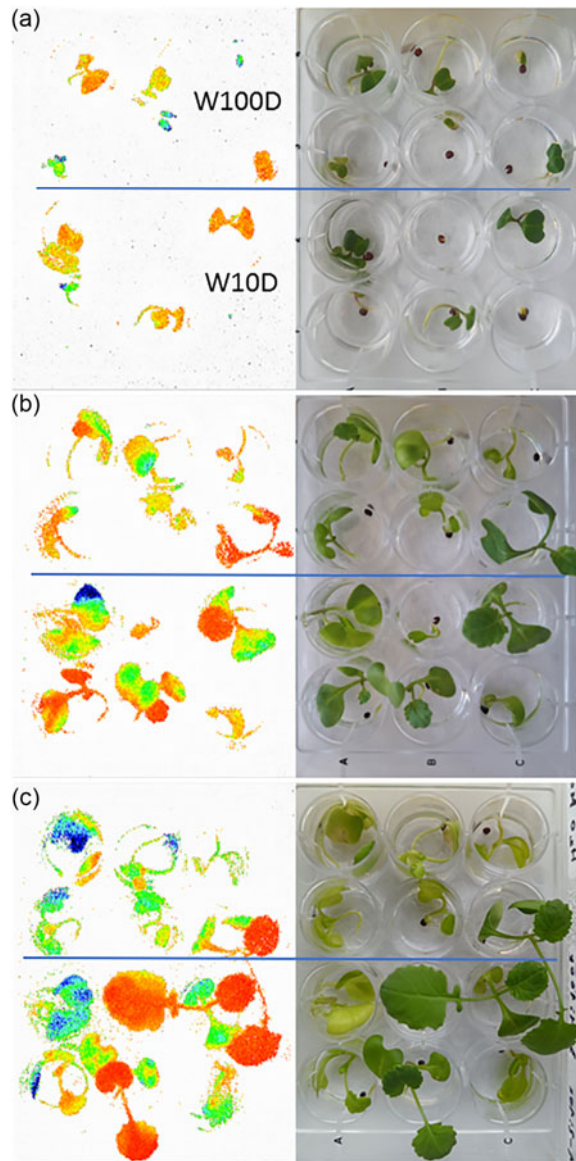


Figure 1. Germination of *B. oleracea* plants in water leachates recorded at the beginning (a), middle (b), and the end (c) of cultivation period. Left column: heterogeneity of chlorophyll fluorescence in germinating plants, the images show F_v/F_m values (pixel colour from low to high: blue-green-yellow-orange-red). Right: photographs of respective germination plants. Treatments: W10D, W100D. Particular treatments are divided by a blue line indicated in the middle of a microbiological plate.

photosynthetic processes. The response of effective quantum yield (Φ_{PSII}) to water and acidic leachates addition was similar to F_v/F_m . There were no statistically significant differences between the treatments and control. Time course of Φ_{PSII} within the experimental cultivation time showed gradual decrease to about 60% of initial value. However, great dilution (W100D, and A100D) led to the lowest Φ_{PSII} values found at the end of experimental cultivation.

No statistically significant decrease in photochemical quenching of chlorophyll fluorescence (qP) was found between untreated control and the treatments exploiting addition of water leachate (W10D,

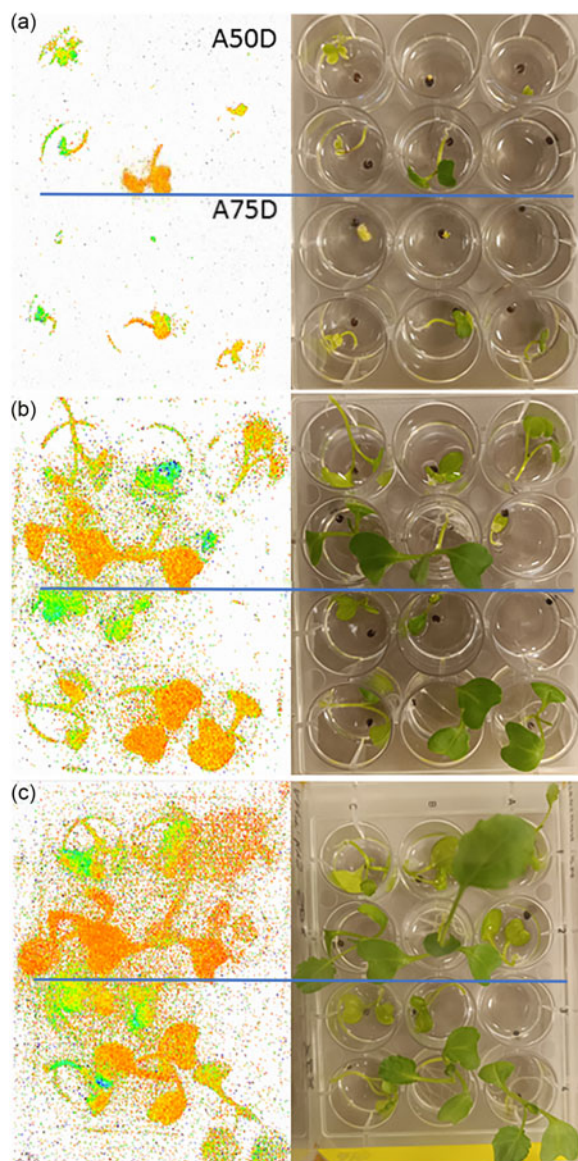


Figure 2. Germination of *B. oleracea* plants in acidic leachates recorded at the beginning (a), middle (b) and the end (c) of cultivation period. Left column: heterogeneity of chlorophyll fluorescence in germinating plants, the images show F_v/F_m values (pixel colour from low to high: blue-green-yellow-orange-red). Right: Photo of respective germination plants. The A50D and A75 treatments (addition of acidic leachates into MS medium) are presented.

W100D). The same was true for the treatments with acidic leachates. There was no difference in qP when compared to control or even within the group of A50D, A75D and A100D treatments. The qP values, irrespective of the treatments showed either no change or declined to only a very limited extent with time of cultivation. Thus, the fraction of open PSII reaction centres remained more or less unchanged by the treatments exploiting the W10D, W100D, A50D, A75D and A100D treatments.

Vitality of germinating plants expressed as relative fluorescence decrease ratio (Rfd) showed a bi-phasic response (Figure 6d). Both in water and acidic leachates added into the MS medium, Rfd showed

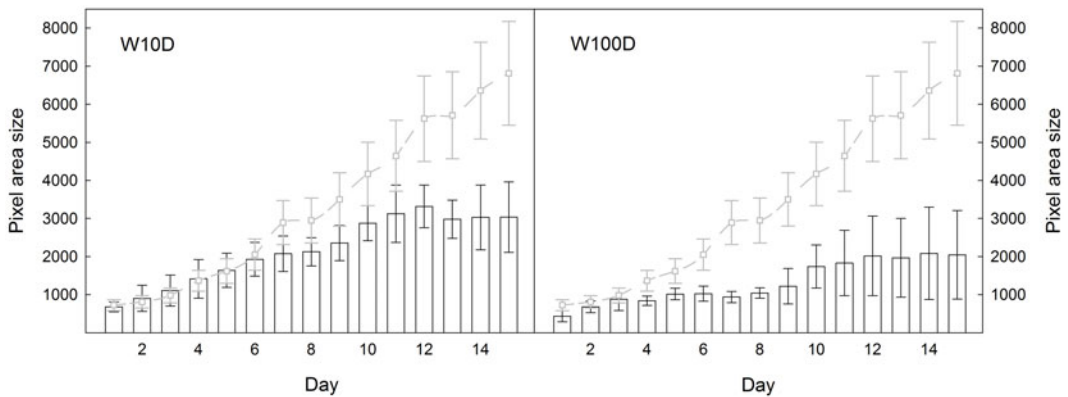


Figure 3. Plant growth of *B. olearcea* as affected by water leachates W10D and W100D (open bars) and in unaffected control (exclusive Murashige-Skoog medium, data points) in the cultivation period. Plant growth is expressed as number of pixels fitting into the area of cotyledons, epicotyl and hypocotyl visualised by chlorophyll fluorescence imaging. Means are presented $\pm$ standard deviations.

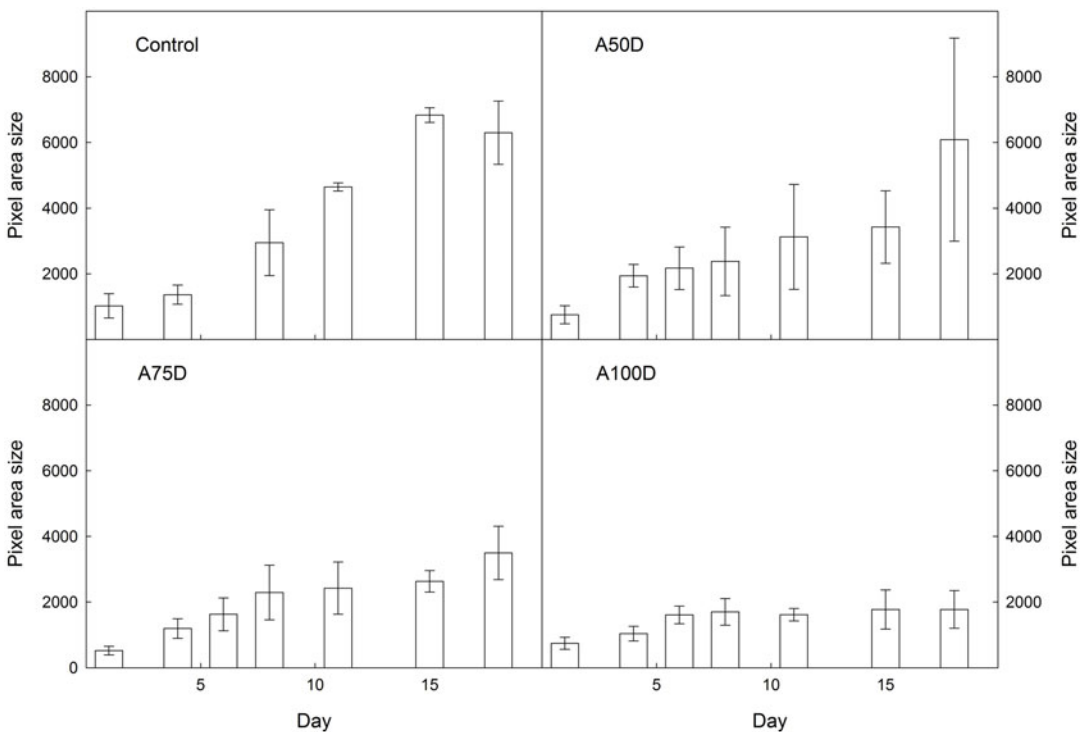


Figure 4. Plant growth of *B. olearcea* as affected by acidic leachates A50D, A75D and A100D in the cultivation period. The treatments affected by A10D, and A25D dilutions did not germinate. Plant growth is expressed as number of pixels fitting into the area of cotyledons, epicotyl and hypocotyl visualised by chlorophyll fluorescence imaging. Means are presented $\pm$ standard deviations.

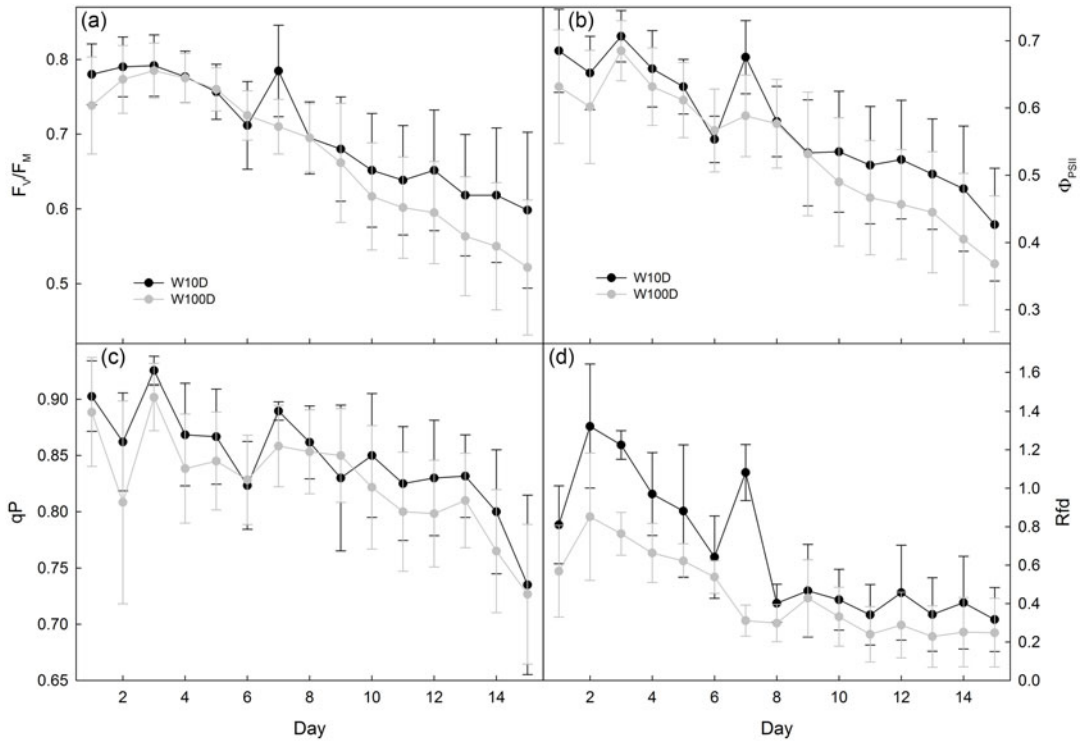


Figure 5. Chlorophyll fluorescence parameters (F_v/F_m – A, Φ_{PSII} – B, qP – C and Rfd – D, for definitions, see Table 4) measured in *B. oleracea* growing in a MS medium with addition of water leachate (W10, W100, i.e., 10 times, and 100 times diluted).

an increase apparent within the first few days of cultivation (day 2 to 6) followed by an approximately constant-rate decrease until the end of cultivation period, when minimum Rfd values were recorded for control (1.1) and water (0.25–0.3) and acidic leachates (0.8–0.1).

Discussion

Acidic leachates had more negative effects on *B. oleracea* germination than water leachates. Acidic leaching of lunar regolith simulant obviously led to an increased content of the metal ions (compared to aqueous leaching) – see Table 2. The metal ions are present in higher amounts in acidic than water extracts (by the factor of 100–100). Therefore, no germination was found in the A10D, and A25D treatments might be attributed to too high concentration of metal ions in medium that caused full limitation of germination. Specifically for broccoli, concentration higher than 1mM of Cd and Ni in growth medium is reported to cause full inhibition of germination (di Salvatore, 2008). Even in the plants that showed successful germination (A50D, A75D and A100D), if cultivated for a longer time (in terms of weeks) under the experimental conditions used in this study, may face negative effects on photosynthesis, vigour and growth rate. The reason is that excessive concentrations of heavy metals inhibit various biochemical pathways and cause activation of antioxidative response (both enzymatic, and non-enzymatic antioxidants, for broccoli see Dawood and Azooz, 2019). Finally, heavy metal ions released from regolith and taken up by experimental plants may be the cause of heavy metabolic imbalance, consequent biomass reduction and plant death (Zornoza, 2002). In plants that have been tested in lunar regolith so far, significant interspecific differences are reported for germination rate (e.g. Rickard and Bode, 2021).

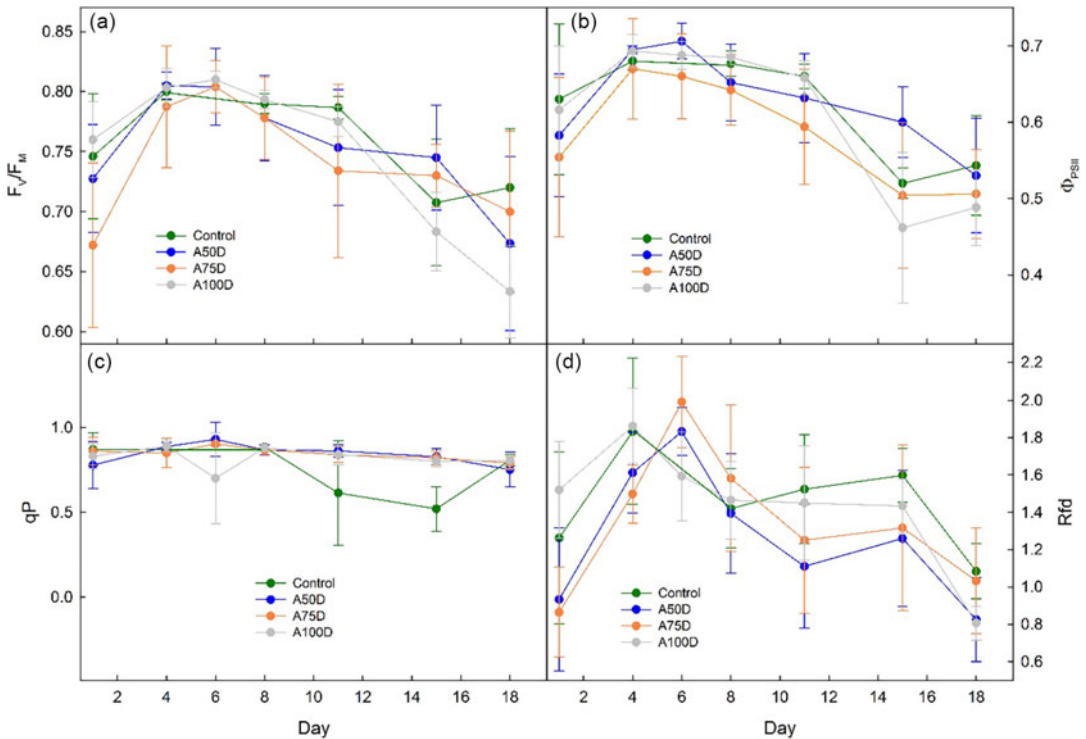


Figure 6. Chlorophyll fluorescence parameters (F_v/F_m – A, Φ_{PSII} – B, qP – C and Rfd – D, for definitions, see Table 4) measured in *B. oleracea* growing in a MS medium with addition of acidic leachate from a lunar regolith simulant (A50, A75, A100, i.e. 50, 75 and 100 times diluted).

Limitation of germination by heavy metal ions is a well-described phenomenon. Critical concentration of heavy metals that fully inhibits germination is species specific, however, the concentrations of Pb and Cu higher than 400 mg L^{-1} are reported to prevent seed germination (Vasilachi-Mitoseru *et al.*, 2023). In this study, suppression of seed germination in the A10D and A25D treatments might be attributed to heavy metal ions effect, especially Zn, Ni and Cr which were present in the leachate in high concentration. Substantial inhibition on seed germination (below 25%) is reported for Zn when the concentration of 100 pp or higher is used (Bezini *et al.*, 2019). Similarly, Astuti *et al.* (2022) reported almost zero germination caused by Cr (100 mg L^{-1}). However, different species show different levels of tolerance to Cr^{2+} . Germination experiment by Jun *et al.* (2009) showed that *Lablab purpureus* might be ranked among most sensitive species to Cr^{2+} , while other tested species (*Lathyrus odoratus*, *Dumasia villosa*) showed no reduction of germination.

Plant growth (see Figure 4) was much more vigorous in control than in the treatments exploiting water and acidic leachates from the lunar regolith simulant. The growth, however, declined with higher degree of the leachate dilution, both in water and acidic leachate. Reason for such reduced growth might be found in bio effectivity of particular concentrations of heavy metal ions in relation to macronutrients (N, P in particular), their uptake and effective utilisation in the emerging plants. This aspect will be addressed in follow up study of germinating plants cultivated in regolith and supplied either by demineralised water or Murashige-Skoog medium (unpublished data, Barták *et al.*, MS in prep).

Rate of plant germination and the early phases of shoot and root growth might be affected by liquid culture itself. In this concept, liquid culture may potentially affect plant seed germination. Root physiology, respiration in particular, might be limited by insufficient oxygen supply in fully submerged roots. It is well established that oxygen is a key factor controlling seed germination (Corbineau, 2022). Prolonged root submersion may lead to asphyxia and disruption of root function and, finally, result in

hypoxia and plant death. During a prolonged hypoxia (typically 3 weeks of fully submerged non-aerated roots), enzymatic activity is altered in order to stimulate alternative N assimilation pathway (Horchani and Aschi-Smiti, 2010). Recent studies focus on different aspects of species-specific tolerance to prolonged hypoxia (Fukao *et al.*, 2003, Kretschmar *et al.* 2015) mainly in relation to waterlogging (for review see Zhou *et al.* 2020). In our experiment, however, root anoxia during the cultivation time was unlikely and physiological limitations caused by insufficient oxygen supply to roots might be excluded since the roots were not fully submerged during the cultivation period (see Material and Methods).

Germination of seeds in liquid in the holes of microbiological plates entails the aspect of missing support for the root and vertical shoot growth. It is very likely that, if provided some support by *e.g.* 1.0–3.0 mm sized glass/ceramic balls, the germinating *B. oleracea* would show more vigorous early growth and leaf area development. It is important to say that our experiments with plants cultivated in fine-grained regolith provided with water or MS proved vigorous growth (Ul Haq *et al.*, 2026). Another limitation of our germination system could be the fact that some leaves remained located on the liquid surface before starting to incline towards the vertical. This might be considered the cause of the relatively small growth inhibition since the leaves showed inability to extend the shoot vertically for a couple of days, however, later, they tended to grow normally (cf. Figure 1a,b).

Apart from the growth of germinating *B. oleracea*, time courses of chlorophyll fluorescence parameters showed dilution-dependent decrease in PSII functioning (cf. F_v/F_m and Φ_{PSII} values for control and W100D, A100D in Figures 5a,b and 6a,b). Reason for such decrease is unclear, however, it may be caused by a complex interaction between light harvesting complexes and reaction centres of PSII, light energy absorption and transfer to photosystem II in particular and activity of oxygen-evolving complex (OEC) attached to the lumen side of the PSII complex, when affected by heavy metal ions. To point out a single reason for heavy metal-induced decline on chlorophyll fluorescence parameters related to PSII is difficult even for specialised biophysical techniques evaluating heavy metals-induced reactive oxygen species and their effect on energy transformation in PSII. General negative impact of heavy metals in PSII is associated with concentration-dependent inhibition of PSII effectivity. Typically, heavy metal effects in PSII lead to a decrease in F_v/F_m and Φ_{PSII} values (Jocsák *et al.*, 2024 for Cd and Zn, Djukic *et al.*, 2014 for Pb, Gong *et al.*, 2019 for Cu, Chen *et al.*, 2018 for Cr), similarly to other stressors, such as high light (Ul Haq *et al.*, 2024), salt stress (Akhter *et al.*, 2021) or the two stressors in combination (Yan *et al.*, 2018). However, the early fluxes of absorbed light energy through PSII studied by fast chlorophyll fluorescence transient (OJIPs and OJIP-derived parameters) respond sensitively to heavy metals in chloroplast (thylakoid membrane) of model algae (*e.g.*, Perrault *et al.*, 2011) and vascular plants (Zhou *et al.*, 2024).

Gradual decrease in chlorophyll fluorescence parameters related to functioning of PSII in chloroplasts is clearly comparable to experimental evidence gained for *B. oleracea* in similarly-designed germination experiment that used Antarctic regolith from James Ross Island (Krejčí *et al.*, 2024). Gradual decline in PSII functioning, specifically Φ_{PSII} might be attributed both to cultivation conditions (Φ_{PSII} declined with time of cultivation even in control plants), and negative effects of heavy metal ions released from a lunar regolith simulant, taken up by emerging plants and allocated in chloroplasts. High content of heavy metal ions in chloroplasts and consequent reactive oxygen species formation are reported for plants grown in lunar (Barcellina *et al.*, 2024) and Martian (Chinnannan, 2023) regoliths or their simulants among the reasons of decreased chlorophyll contents in leaves. Oxidative stress, and activation of antioxidative mechanisms, might be considered one of the most important physiological response of photosynthetic tissues in plants cultivated in lunar regolith and its analogues. It was shown by Paul *et al.* (2022) that the plants grown in lunar regolith do activate a series of stress response pathways, most notably the oxidative stress response. Moreover, heavy metals-induced alterations in nutrient uptake rate from MS cultivation medium might be considered another co-acting factors. In acetic leachate, Zn represented a major element. Therefore, Zn-induced interactions with the PSII photosynthetic apparatus might be taken into consideration because heavy metals-induced inhibition of PSII functioning is reported for a variety of plants (Qin *et al.*, 2023).

Concluding remarks

This study revealed that acidic leachates from lunar regolith simulant, when added to Murashige-Skoog cultivation medium, caused a more pronounced inhibition of *Brassica oleracea* germination compared to water leachates leading to complete suppression of seed germination. Even when diluted 10- or 25-fold, the acidic leachate continued to inhibit germination. In contrast, undiluted leachates from the simulant, both acidic and water, entirely suppressed the seed germination. On the other hand, water leachates did not show such detrimental effects. *B. oleracea* exhibited satisfactory germination when treated with a 10-fold diluted water leachate. This suggests that water leachates from lunar regolith or its simulants could be viable for future studies aimed at cultivating plant species in such media. In support of this, a recent study by our team (MS in prep.) on the growth of *B. oleracea*, *Raphanus sativus* and *Capsicum annuum* in Antarctic regolith without added organic matter (for regolith characteristics, see Coufalík *et al.* 2024) showed successful plant growth and adequate photosynthetic performance, even when irrigated solely with demineralised water. However, the aboveground growth and development were less vigorous compared to plants watered with Murashige-Skoog medium.

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