

Article

Ion-Exchanged Clinoptilolite as a Substrate for Space Farming

Yuri Kalvachev ¹, Ermenegilda Vitale ², Carmen Arena ², Totka Todorova ¹, Daniel Ilkov ³
and Violeta Velikova ^{3,4,*}

- ¹ Institute of Catalysis, Bulgarian Academy of Sciences, “Acad. G. Bonchev” Str., bl. 11, 1113 Sofia, Bulgaria; kalvachev@ic.bas.bg (Y.K.); t.todorova@ic.bas.bg (T.T.)
- ² Department of Biology, University of Naples Federico II, Via Cinthia, 80126 Naples, Italy; ermenegilda.vitale@unina.it (E.V.); carena@unina.it (C.A.)
- ³ Institute of Plant Physiology and Genetics, Bulgarian Academy of Sciences, Acad. G. Bonchev Str., bl. 21, 1113 Sofia, Bulgaria; d_ilkov@bio21.bas.bg
- ⁴ Institute of Biophysics and Biomedical Engineering, Bulgarian Academy of Sciences, Acad. G. Bonchev Str., bl. 21, 1113 Sofia, Bulgaria
- * Correspondence: violet@bio21.bas.bg

Abstract: Clinoptilolite, with its structural peculiarities (ion-exchange and adsorbent properties), is an excellent candidate for direct use and various modifications. In this study, we explored the effect of ion exchange and the particle size of clinoptilolite on *Raphanus sativus* seed germination, plant growth, physiological and biochemical characteristics of plants. Plants were grown, for three consecutive runs, on non-modified clinoptilolite, 0.9–2.5 mm (C-2.5) and 2.5–5.0 mm (C-5.0); clinoptilolite fractions modified with ion exchange with ammonium (C_{NH4}-2.5 and C_{NH4}-5.0); and potassium (C_K-2.5 and C_K-5.0) ions. Our data revealed that ion exchange with ammonium increased water-holding capacity, while potassium exchange decreased the water-holding capacity of the substrates irrespective of their particle size. The positive effect of small fractions ion-exchanged clinoptilolite (C_{NH4}-2.5 and C_K-2.5) on seed germination, during the third run, was established. The small clinoptilolite fractions favored root crop production, particularly in C_K-2.5 plants only during the first run. Substantial positive effect on the content of total carbohydrates and polyphenols especially during the third run was established in plants grown on potassium-exchanged clinoptilolite. Our findings support the future exploration of clinoptilolite as a suitable substrate for plant growth in space and ground-based facilities for space-oriented experiments.

Keywords: natural zeolites; charge-compensating cations; growth medium; seed germination; plant development; physiological and biochemical response



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1. Introduction

The colonization of planets other than Earth is one of the biggest science challenges of humans. As technology has advanced, human settlement on the Moon or Mars is becoming more and more realistic. Several programs supported by different countries or private agencies have already announced human establishment on the Moon or Mars in the next ten–twenty years. Colonization on planets other than Earth, however, is not simple, and there are many obstacles to overcome. One of the major issues is food availability [1]. There are various options to grow food on Mars or the Moon, such as inner substrates with a nutrient solution as the medium, or aeroponics [2]. Hydroponic and aeroponic growing systems, however, are not capable of absorbing the inedible part of plants. For this reason, there is continuously increasing interest in “soil-based” space agriculture, particularly in developing solid substrates for supplying nutrients during long-term missions in a space habitat [3]. Within planning of the life-support systems for regenerating resources, i.e., air, water and food, the Johnson Space Center (JSC) of the National Aeronautics and Space Administration (NASA) has devised a zeolite-based synthetic substrate consisting of synthetic apatite and clinoptilolite, whose cations are exchanged for NH₄⁺ and K⁺ cations,

representing the principal available nutrients for plants [4,5]. Unsurprisingly, a spectral analysis suggested that components of the martian regolith are more likely zeolites [6–9], and clinoptilolite is considered one of the most widespread zeolites on Mars [10].

Zeolites are hydrated crystal aluminosilicates with porous structures. The primary building unit of the zeolite framework is a tetrahedron, the center of which is occupied by silicon or aluminum atoms, with four oxygen atoms at the vertices. Substitution of Si^{4+} with Al^{3+} provides a negative charge for the framework, which is balanced by monovalent or bivalent cations located at the surface. Ions can be easily exchanged with other ions, and zeolites possess ion-exchange features, making them unique materials that can be contributors to a variety of properties. The aluminosilicate framework, defining the structure type, is the most stable component. In natural zeolites, the molar ratio of silicon to aluminum (Si/Al) in the crystal is in the range of 1 to 6. The Löwenstein rule determines the lower limit of the Si/Al ratio. According to this rule, AlO_4 tetrahedrons cannot be combined with other tetrahedron AlO_4 using mutual oxygen atoms and at the Si/Al ratio = 1 where AlO_4 and SiO_4 tetrahedrons alternately form the mineral skeleton [11]. The lowest Si/Al ratio in the zeolite framework is 1. The molar ratio of Si/Al is an important parameter that determines ion-exchange capacity, hydrophilicity, thermal and acid stability, acid properties in the case of H-form, etc.

There are four ion-exchange centers in the structure of clinoptilolite, according to the structural model of Koyama and Takeuchi [12], and usually they are preferentially occupied by the ions existing in nature— Ca^{2+} , Na^+ , K^+ , Mg^{2+} —and every ion selectively occupies certain structural positions located in the channel system of the zeolite with different free volume around them. The selectivity of the exchange depends on the initially present ions in the composition of the zeolite, as well as on the degree of hydration of these ions.

Zeolites have cation-exchange properties, but their tetrahedral framework is rigid, and the size of the pores is fixed. Therefore, small cations can move freely into and out of the pores, while larger cations are excluded. For this reason, zeolites are often called ‘molecular sieves’ because of their very selective cation-exchange properties [13].

Clinoptilolite is widespread and abundant in nature. This mineral has a volcanic origin, and it is derived from lava, ash and water. The world’s annual production of natural zeolite is about 3 million tons, and more than 80% of the world’s zeolite production is clinoptilolite. Rich deposits of clinoptilolite are present in the Eastern Rhodope Mountains of Bulgaria.

Zeolites, in general, are known for their beneficial properties for creating substrates for plants growth. Petrov et al. [14] patented a composition of a substrate for growing plants in greenhouses, which includes zeolite clinoptilolite, vermiculite and peat in certain proportions and particle size limits, with increased content of nitrogen and phosphorus. The patented material provides flexible and active plant growth in both the greenhouse and outdoors. The potential application of natural zeolites as soil conditioners, slow-release fertilizers, as agents against insecticides and pesticides, or for remediation in contaminated soils have been also investigated [15–17]. Natural and synthetic zeolites have high water-holding capacity and can lose and gain water reversibly, without any changes in their crystal structure. Experiments with zeolites synthesized from a cheap source, such as coal fly ash, indicated that zeolite addition increases the water-retention capacity of soil and decreases the drainage capacity. These effects are mainly attributed to the deviation of the initial pore size distribution of the amended substrates that reassembled their internal structure [16].

Although numerous potential applications have been proposed, there is a need for a complete characterization of the zeolites in terms of particle size, chemical composition after ion exchange and long-lasting release of nutrients. Furthermore, additional information is required about the occurrence of zeolite–plant interaction and the suitability of this substrate to be reutilized for consecutive plant growth cycles.

In the present work, we aim to investigate the effect of the particle size and ion exchange of clinoptilolite on *Raphanus sativus* seed germination, growth, development, and

the physiological and biochemical characteristics of plants utilizing the same substrate for three cultivation cycles (first, second and third runs). To assess the effects of a growth medium on seed germination and plant development, the following scenarios were used: clinoptilolite with granulous size of 0.9–2.5 mm and 2.5–5.0 mm and modified clinoptilolite with ammonium and potassium ions. Here, different well-known techniques in the structural and chemical characterization of zeolites as well as plant physiology were applied. The measurements revealed for the first time that the ion-exchange process affects the water-holding capacity of the zeolite, and these changes are not related to the particle size. The small fraction (0.9–2.5 mm) is more suitable for growing radish compare to bigger one (2.5–5.0 mm). Ion-exchanged clinoptilolite had positive effect on seed germination, biomass production, and carbohydrate and polyphenols content of root crop especially in the third run. Our findings contribute to better understanding of the use of clinoptilolite as a growth medium that meets specific chemical, biological and agronomic requirements for plant development in space as well as in ground-based facilities for space-oriented experiments.

2. Materials and Methods

2.1. Clinoptilolite Ion Exchange

The ammonium and potassium forms of clinoptilolite were obtained through treatment of zeolite with 1 M solution of ammonium nitrate and potassium nitrate, respectively, at 80 °C for 24 h and subsequent filtration, washing and drying at 80 °C. The utilized substrates are listed in Table 1.

Table 1. Abbreviations of the utilized non-ion-exchanged and ion-exchanged clinoptilolite fractions.

non-ion-exchanged clinoptilolite with zeolite particle size in the range 0.8–2.5 mm	C-2.5
non-ion-exchanged clinoptilolite with zeolite particle size in the range 2.5–5.0 mm	C-5.0
ion-exchanged clinoptilolite with ammonium nitrate with zeolite particle size in the range 0.8–2.5 mm	C _{NH4} -2.5
ion-exchanged clinoptilolite with ammonium nitrate with zeolite particle size in the range 2.5–5.0 mm	C _{NH4} -5.0
ion-exchanged clinoptilolite with potassium nitrate with zeolite particle size in the range 0.8–2.5 mm	C _K -2.5
ion-exchanged clinoptilolite with potassium nitrate with zeolite particle size in the range 2.5–5.0 mm	C _K -5.0

2.2. Clinoptilolite Characterization

The X-ray powder diffraction patterns were recorded on a diffractometer D2 Phaser (Bruker, Billerica, MA, USA) with CuK α radiation, working at an acceleration of 30 kV and current of 10 mA. The 2 θ scanned range was 4–40 degree with increments of 0.05 degree min⁻¹ and 1 s of acquisition time.

FTIR spectra were taken on a Bruker Tensor 37 spectrometer using a KBr pellet technique. For each sample, 64 scans were collected at a resolution of 2 cm⁻¹ over the wavenumber region 4000–400 cm⁻¹. Elemental analyses were conducted using an X-ray fluorescence spectrometer, Rigaku Simultix (Rigaku Europe SE, Neu-Isenburg, Germany), and previous loss in heating (1000 °C) were determined using weight analysis.

The water-holding capacity of non-ion-exchanged and ion-exchanged clinoptilolite fractions was defined gravimetrically as in [18]. Briefly, air-dried clinoptilolite samples were placed in a cylinder filled with sufficient water for 72 h to completely saturate the substrate. Then, the zeolite samples were removed from the water and let to drip until the dripping of the water stopped, and the weight of the saturated samples was measured. Then, the samples were dried in an oven at 105 °C to a constant weight.

2.3. Plant Experiments

Raphanus sativus plants were grown from seeds in a climate chamber under controlled conditions: day/night temperature 25/20 \pm 3 °C air temperature, relative humidity 65%, light intensity 400 μ mol m⁻² s⁻¹ photosynthetic photon flux density (PPFD), and a 14 h photoperiod. The following scenarios of growth mediums were applied: two fractions

of non-modified clinoptilolite 0.9–2.5 mm (C-2.5) and 2.5–5.0 mm (C-5.0); clinoptilolite fractions modified with ion exchange with ammonium ($C_{NH_4-2.5}$ and $C_{NH_4-5.0}$); and potassium ($C_K-2.5$ and $C_K-5.0$) ions. In order to understand the possible changes in the substrate properties after the first use, two more complete cultivation cycles on the utilized substrates were carried out.

Seeds were placed on the wet (80% of full water capacity) substrate in the dark for two days. Then, the light was switched on and the seed germination was checked every 24 h for a period of 4 days. Measurements of chlorophyll fluorescence were carried out 15 days after the seed sowing. Then, plants were grown for an additional 45 days to allow the development of the root crop. At the end of this period, the plants were either used for fresh or dry biomass determination, and root samples were collected for further biochemical analyses. Dry biomass accumulation was determined after leaf and root drying at 105 °C for 30 min and then at 60 °C for constant weight.

2.4. Chlorophyll Fluorescence and Leaf Pigment Measurements

Modulated chlorophyll fluorescence was measured using IMAGING-PAM MAXI version (H. Walz GmbH, Effeltrich, Germany) supplemented with a blue excitation light unit (IMAG-MAX/L LED) and IMAG-K7 CCD camera. The measurements were carried out on intact plants after 30 min of dark adaptation followed by a saturating pulse (over 6000 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ PPFD) with a duration of 0.8 s to determine the minimum (F_0) and maximum (F_m) fluorescence and to calculate the maximum photosystem II (PSII) quantum yield [$F_v/F_m = (F_m - F_0)/F_m$]. Leaves were then adapted to the specific actinic light (180 $\mu\text{mol m}^{-2} \text{s}^{-1}$ PPFD) and saturating pulses were applied. This allowed us to determine the steady-state fluorescence (F') and the maximum fluorescence (F_m') of the light, and to calculate the quantum efficiency of PSII in illuminated leaves [$\Phi_{PSII} = (F_m' - F')/F_m'$] [19] and the non-photochemical quenching [$NPQ = (F_m - F_m')/F_m'$] [20].

Leaf pigments' (total chlorophyll and flavonols) abundance was estimated using a multi-pigment meter (MPM-100, Opti-Sciences Ins., Hudson, NH, USA).

2.5. Biochemical Analyses

Dry samples preventively powdered in liquid nitrogen were used to determine the total protein, carbohydrate and polyphenol content using colorimetric assays. All analyses were carried out with three replicates per treatment. The spectrophotometric measurements were performed using a microplate reader spectrophotometer (BioTek Synergy HTX, Santa Clara, CA, USA, Software: Gen5TM 2.07).

2.5.1. Soluble Proteins

The total soluble protein concentration was estimated following the methods of Bradford [21] and Im et al. [22]. Samples (50 mg) were homogenized in 0.2 M potassium phosphate buffer (pH 7.8 + 0.1 mM EDTA) and then centrifuged at 10,000 rpm for 20 min at 4 °C. The supernatant was mixed with the dye reagent to read the absorbance at 595 nm. The total soluble protein concentration was retrieved using a bovine serum albumin (BSA) calibration curve and expressed as the mg BSA equivalent per gram of dry weight (mg BSA eq g^{-1} DW).

2.5.2. Carbohydrates

The total carbohydrate content was determined by applying the anthrone method described by Hedge and Hofreiter [23] and slightly modified as in Vitale et al. [24]. Samples (10 mg) were treated with 2.5 N HCl to hydrolyze carbohydrates into simple sugars and determine the carbohydrate concentration through a colorimetric reaction, employing the anthrone reagent dissolved in ice-cold H_2SO_4 . The absorbance was measured at 630 nm, and the total sugars were measured with a glucose standard curve and then expressed as mg glucose equivalents per gram of dry weight (mg Glu eq g^{-1} DW).

2.5.3. Polyphenols

The total polyphenols were measured following Vitale et al. [25]. Samples (50 mg) were extracted using methanol and centrifuged at 11,000 rpm for 5 min. Extracts were added to 10% Folin–Ciocalteu (1:1, *v/v*), shaken, and after 3 min, mixed with 700 mM Na₂CO₃ solution (5:1, *v/v*). Samples were incubated for two hours in darkness, and the absorbance was read at 765 nm. The total polyphenol content was obtained from a gallic acid calibration curve and expressed as mg of gallic acid equivalents per g of dry weight (mg GA eq. g⁻¹ DW).

2.5.4. Statistical Analysis

All data were analyzed using the SigmaPlot 12 software (Jandel Scientific, San Rafael, CA, USA) applying the one-way ANOVA. The Kolmogorov–Smirnov test was used to check the normality. The Student–Newman–Keuls (SNK) test was applied for all pairwise multiple comparison tests with a significance level of $p < 0.05$.

3. Results and Discussion

3.1. Effects of Clinoptilolite Ion Exchange on the Structure and Chemical Composition of Zeolite

In Figure 1, X-ray patterns of non-exchanged and ammonium- and potassium-exchanged clinoptilolite from Bentonite AD (Beli Plast, Eastern Rhodope Mountains, Bulgaria) are presented. There are no differences between the presented X-ray patterns—all of them contain typical X-ray reflexes for clinoptilolite [12]. This fact shows that there are no detectable crystalline structure changes during the ion-exchange process.

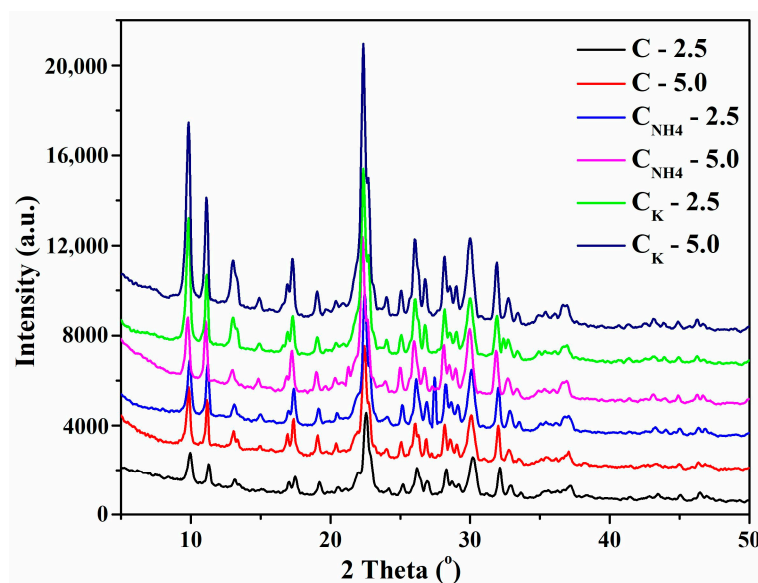


Figure 1. X-ray patterns of investigated substrates.

Table 2 presents the chemical analysis of the utilized substrates in this investigation after each run of planting.

When clinoptilolite was exchanged with ammonium ions, it is seen that these ions replace both the monovalent potassium and sodium ions and the divalent calcium ions. During this process the replacement of sodium and calcium ions was about 60–65%, while for potassium, it was 10–15%, and it was only 6–7% for magnesium. This was observed for both the investigated substrates, C-2.5 and C-5.0.

During the exchange with potassium nitrate, the sodium ions were completely substituted and up to 60–70% of the calcium ions were displaced from the potassium ions. In this treatment with potassium nitrate, magnesium ions were replaced to a greater extent than in the process of ion exchange with ammonium nitrate. The resulting clinoptilolite contained twice as much potassium as the starting material. The degree of exchange with potassium

ions was greater than with ammonium ions, which is expected considering the size of the ions. The ammonium ion is larger and does not reach all the ion-exchange centers in clinoptilolite unlike the potassium ion.

Table 2. Chemical analysis of utilized substrates on the base of clinoptilolite with expanded uncertainty $\pm 0.05\%$.

Clinoptilolite (C): 0.8–2.5 mm/wt. %							
Sample	Al ₂ O ₃	CaO	Fe ₂ O ₃	MgO	K ₂ O	Na ₂ O	SiO ₂
C-2.5	11.74	3.49	0.87	0.89	2.91	0.55	71.42
C-2.5 after 1st run	12.00	3.42	0.84	0.90	2.94	0.46	71.31
C-2.5 after 2nd run	11.80	3.45	0.84	0.93	2.88	0.45	71.23
C-2.5 after 3rd run	11.85	3.48	0.85	0.95	2.82	0.44	71.48
C _{NH4} -2.5	11.85	1.61	0.85	0.77	2.67	0.22	71.68
C _{NH4} -2.5 after 1st run	11.74	1.80	0.85	0.82	2.59	0.32	71.54
C _{NH4} -2.5 after 2nd run	11.84	1.84	0.84	0.80	2.48	0.17	71.59
C _{NH4} -2.5 after 3rd run	11.81	1.78	0.86	0.81	2.46	0.23	71.40
C _k -2.5	11.32	1.38	0.80	0.70	7.40	≤0.01	71.38
C _k -2.5 after 1st run	11.25	1.44	0.93	0.74	7.23	≤0.01	71.35
C _k -2.5 after 2nd run	11.46	1.38	0.82	0.73	7.14	≤0.01	71.41
C _k -2.5 after 3rd run	11.57	1.34	0.85	0.75	7.08	≤0.01	71.33
Clinoptilolite (C): 2.5–5.0 mm/wt. %							
Sample	Al ₂ O ₃	CaO	Fe ₂ O ₃	MgO	K ₂ O	Na ₂ O	SiO ₂
C-5.0	11.72	3.33	0.83	0.81	3.39	0.69	71.53
C-5.0 after 1st run	11.89	3.63	0.85	0.81	3.14	0.70	71.05
C-5.0 after 2nd run	11.72	3.22	0.81	0.72	3.03	0.58	72.08
C-5.0 after 3rd run	11.89	3.36	0.83	0.74	3.05	0.50	71.21
C _{NH4} -5.0	11.98	1.41	0.86	0.87	2.98	0.44	71.09
C _{NH4} -5.0 after 1st run	11.76	1.51	0.87	0.77	2.90	0.35	71.22
C _{NH4} -5.0 after 2nd run	11.78	1.73	0.84	0.75	2.81	0.36	71.30
C _{NH4} -5.0 after 3rd run	11.89	1.55	0.86	0.72	2.58	0.25	71.29
C _k -5.0	12.03	1.16	0.92	0.68	10.58	0.08	70.25
C _k -5.0 after 1st run	11.77	1.44	1.06	0.79	10.12	0.05	69.60
C _k -5.0 after 2nd run	11.70	1.01	0.94	0.66	8.14	0.04	71.18
C _k -5.0 after 3rd run	11.67	1.09	0.83	0.62	8.17	≤0.01	71.23

Interestingly, in the case of a coarse-grained sample with a particle size in the range of 2.5–5.0 mm, the extent of replacement by potassium was higher compared to that of the sample with a smaller particle size. Most likely, the structural positions occupied by potassium are more accessible in the case of the substrate with a larger particle size. In all investigated cases, the amount of potassium decreases in the process of each subsequent sowing.

Beside the high and selective cation-exchange capacity of zeolites, they are also characterized by water holding. As expected, the bigger fraction of the substrates (with a particle size in the range of 2.5–5.0 mm) had lower water-holding capacity compare to the small fraction (with a particle size in the range of 0.8–2.5 mm) (Figure 2). It is interesting to note that both fractions of ammonium-exchanged substrates possess higher water-holding capacity in respect to the non-exchanged substrates. On the contrary, potassium-exchange

decreased the water-holding capacity of the substrates irrespective of their particle size. We speculate that the higher degree of water holding of the substrate ion-exchanged with ammonium ions could be due to the formation of additional hydrogen bonds of the water molecules with the hydrogen from the ammonium ion, as indicated in [26].

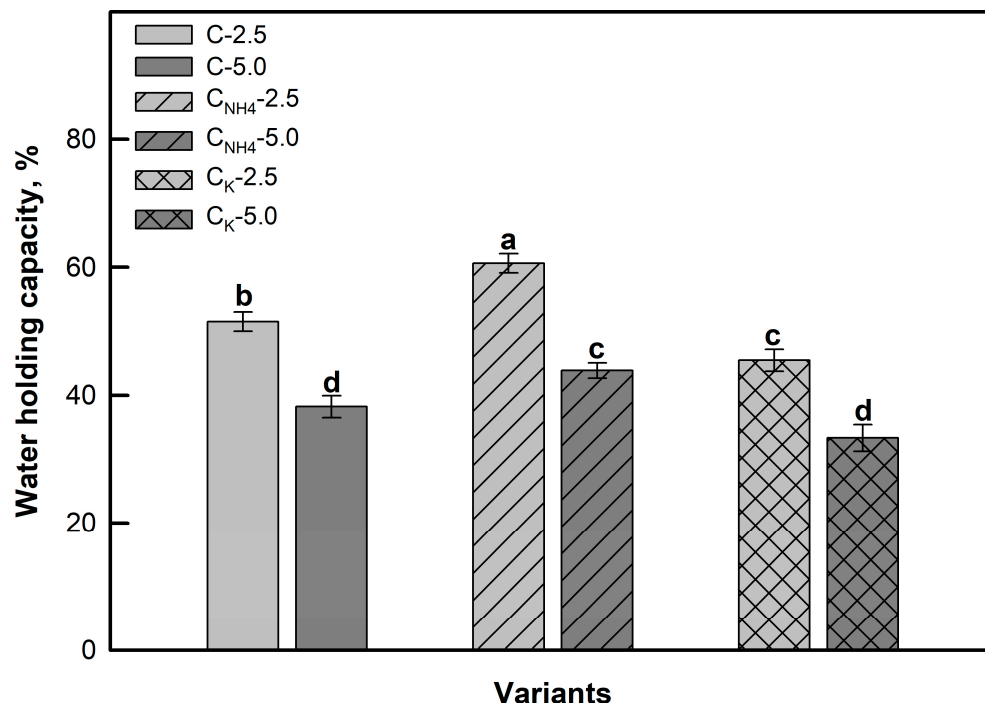


Figure 2. Water-holding capacity of non-modified clinoptilolite (C-2.5 and C-5.0), and clinoptilolite modified with ion exchange with ammonium (C_{NH4}-2.5 and C_{NH4}-5.0) and potassium (C_K-2.5 and C_K-5.0) ions. Means \pm SE (n = 3). One-way ANOVA followed by post hoc Tukey's test were applied, and statistically significant differences are indicated using different letters at $p < 0.05$.

3.2. Effects of Clinoptilolite Particle Size and Ion-Exchange Modification on Seed Germination and Plant Growth

The radish (*Raphanus sativus* L.) was widely used as a model plant for space flight experiments and ground-based studies, because of a series of suitable characteristics including a short cultivation cycle, good production of bioactive compounds and important minerals [27]. The seed germination percentage and the seedling biomass are traits provide important information about the suitability of clinoptilolite to be utilized as a growth substrate. Regarding the effects of particle size and the ion exchange of clinoptilolite on seed germination, the results are illustrated in Figure 3A. The germinability was higher for C_{NH4}-2.5 compared to C-2.5, and the difference was statistically significant when compared to C_K-2.5 during the first run. The differences between ion-exchange substrates were less pronounced for the bigger fraction (first run). No important changes in seed germination were observed for C-2.5 and C-5.0 during the second run, while seed germinability decreased in C-5.0 during the third run, although these changes were not significant. A dramatic reduction in seed germination was established only in C_{NH4}-5.0, while the germination was similar in C_{NH4}-2.5 during the second and third run. C_K-2.5 had a positive effect on seed germination with the timing of the experiment, being significantly higher during the third run.

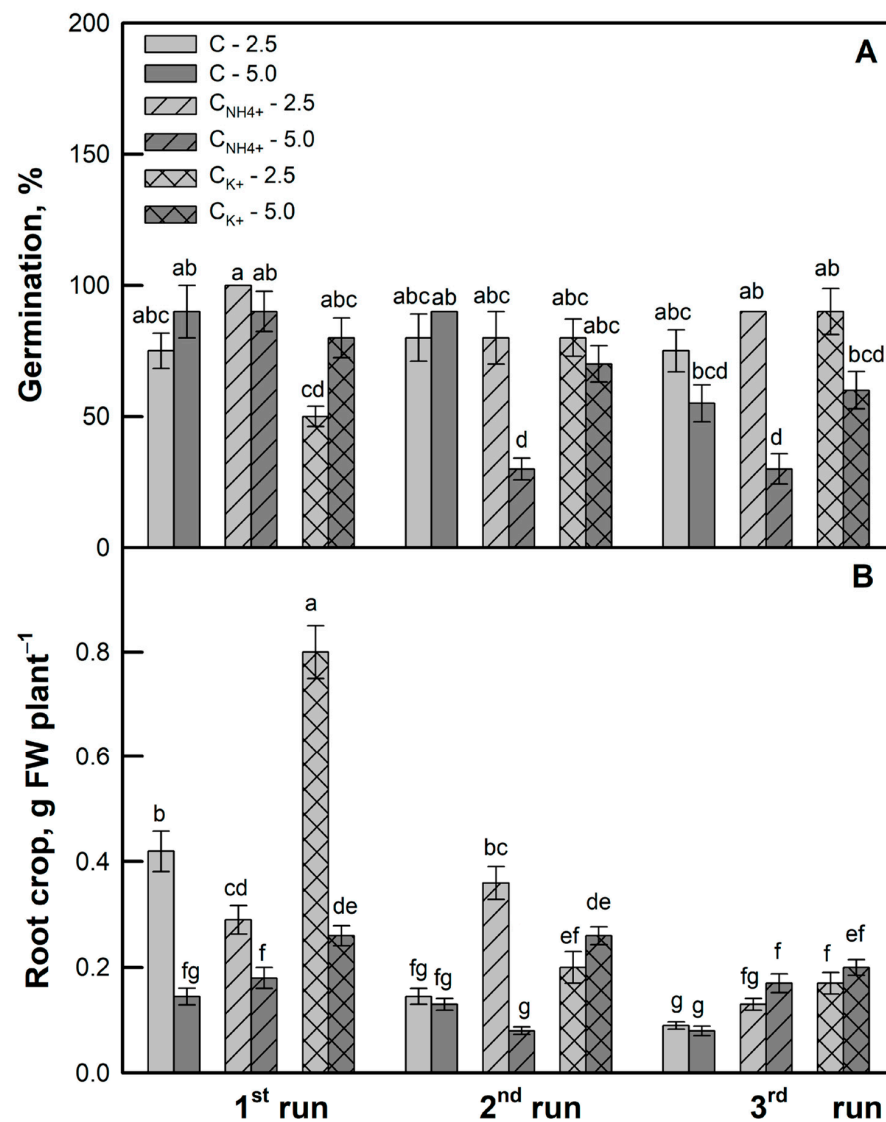


Figure 3. Germination (A) and root crop production (B) for *Raphanus sativus* grown on two fractions of non-modified clinoptilolite (C-2.5 and C-5.0), and clinoptilolite modified with ion exchange with ammonium (C_{NH4+}-2.5 and C_{NH4+}-5.0) and potassium (C_{K+}-2.5 and C_{K+}-5.0) ions. The 1st run was performed on non-utilized substrates, and 2nd and 3rd runs are complete cultivation cycles on the utilized substrates. Means \pm SE (n = 3). One-way ANOVA followed by post hoc Tukey's test were applied, and statistically significant differences are indicated using different letters at $p < 0.05$.

Similarly to germination, root crop production was also favored by the small clinoptilolite fraction compared to the bigger; in particular, the root crop reaches its highest values in its C_{K+}-2.5 plants during the first run (Figure 3B). The utilized substrates negatively affected the root crop with biomass accumulation being more pronounced during the third run. The only exceptions were found for C_{NH4+}-2.5 and C_{K+}-5.0 plants (second run), where no significant changes were observed. The substrate particle size may greatly influence root growth, as it affects the physical and chemical properties of the substrate, such as the structure, oxygen concentration, water content and nutrient availability. According to Huang et al.'s [28] findings, biomass, rhizome length, and tuber number of a rhizomatous wetland plant increased in substrates with medium-size quartz particles corresponding to 1.5 mm compared to larger particles of 3 mm that may exert higher mechanical resistance to root expansion. Similarly, carrot plants treated with the same concentrations of nutrient solutions and grown in 1.2–2.5 mm perlite particles showed longer and heavier roots than those grown on 5 mm particles. Conversely, using a supplemented arcillite substrate with

fine, smaller particles (1–2 mm), the radish roots were shorter, hampered by excessive water retention and oxygen deficiency [29]. In our study, the positive effect of the 2.5 mm particle size is emphasized using potassium nitrate, especially in the first run. Such an effect may depend on the slow-release fertilizer property of clinoptilolite, because of its chemical structure and ion-exchange property. Clinoptilolite zeolite is highly selective for K^+ and may store such nutrients in its structural channels, reducing their leaching. Then, clinoptilolite may allow gradual water and nutrient absorption by plants, thereby increasing shoot and root plant growth [30]. Rahimi et al. [31] recently demonstrated that potassium-enriched clinoptilolite improved the shoot and root biomass in perennial ryegrass plants even under salinity stress. Baninasab [32] confirmed the clinoptilolite fertilization property is able to increase the diameter and fresh weight of edible roots of radish plants. Noori et al. [33] reported zeolites' positive effect on radish growth cultivated under salinity. More attention is required towards the general decrease in the root fresh weight in the third compared to previous runs. Gruener et al. [5] evidenced a progressive increment of the fresh weight of edible roots in radish plants grown in zeoionic substrates even after three successive crops of growth, with 52% of the original NH_4-N and 78% of the original K remaining on zeolite-exchange sites. It is supposable that such kinds of substrates are reservoirs for slow-releasing nutrients in the long-term, and it might explain why, in the third run, we observed an increment of root fresh weight in plants grown in clinoptilolite with ammonium and potassium nitrate compared to the non-modified control. Our results support the earlier findings that clinoptilolite longevity in delivering nutrients is convenient for supplying the crew with fresh food during long-term space missions [5] or in stable stations, employing Martian clinoptilolite as the principal substrate.

3.3. Effects of Clinoptilolite Particle Size and Ion-Exchange Modification on Photochemistry of Photosynthesis and Leaf Pigment

Chlorophyll fluorescence imaging on intact plants was utilized to evaluate the photosynthetic activity alteration as a result of the particle size of the clinoptilolite- and ion-exchange procedure. The values of the maximal efficiency of photosystem II in dark-adapted plants (F_v/F_m) were not significantly different among the studied variants (Figure 4A). The actual photochemical activity of photosystem II in the light-adapted state (Φ_{PSII}) did not significantly vary among the treatments during the first and second runs (Figure 4B). It should be noted that $C_K-2.5$ plants were characterized with significantly higher Φ_{PSII} compared to $C-2.5$ (third run), which could be due to the improved K-nutritional status of plants, contributing to maintaining the electron flow in photosynthesis [31].

A significant increase in non-photochemical quenching (NPQ) parameters was registered only in $C_{NH_4}-5.0$ plants during the first run (Figure 4C). A more pronounced rise in NPQ was detected in $C-2.5$, $C-5.0$ and $C_K-2.5$ during the second run, suggesting an activation of photoprotective mechanisms in those plants, while no important changes were registered in the rest of the variants. During the third run, the tendency for NPQ reduction was established in most of the plants, suggesting that consecutive plant growth on different fractions of ion-modified clinoptilolite did not hamper the effectiveness of plant protection from photodamages.

The chlorophyll content of African marigold plants was improved using zeolite, which increases water retention in soil and prevents the leaching of elements such as nitrogen [30]. Higher chlorophyll content was also observed in strawberry plants treated with ammonium-enriched zeolite [34]. However, in our experiments the total chlorophyll content remained similar during the first run (Figure 5A). The re-used substrates did not cause significant fluctuation in the chlorophyll level independently of the particle size and ion-exchange procedure. Probably, the absence of significant variations may depend on the unchanged SiO_2 concentrations observed during the three growth cycles, as, based on previous studies, Si positively affects leaf chlorophyll content, preventing pigment degradation [35].

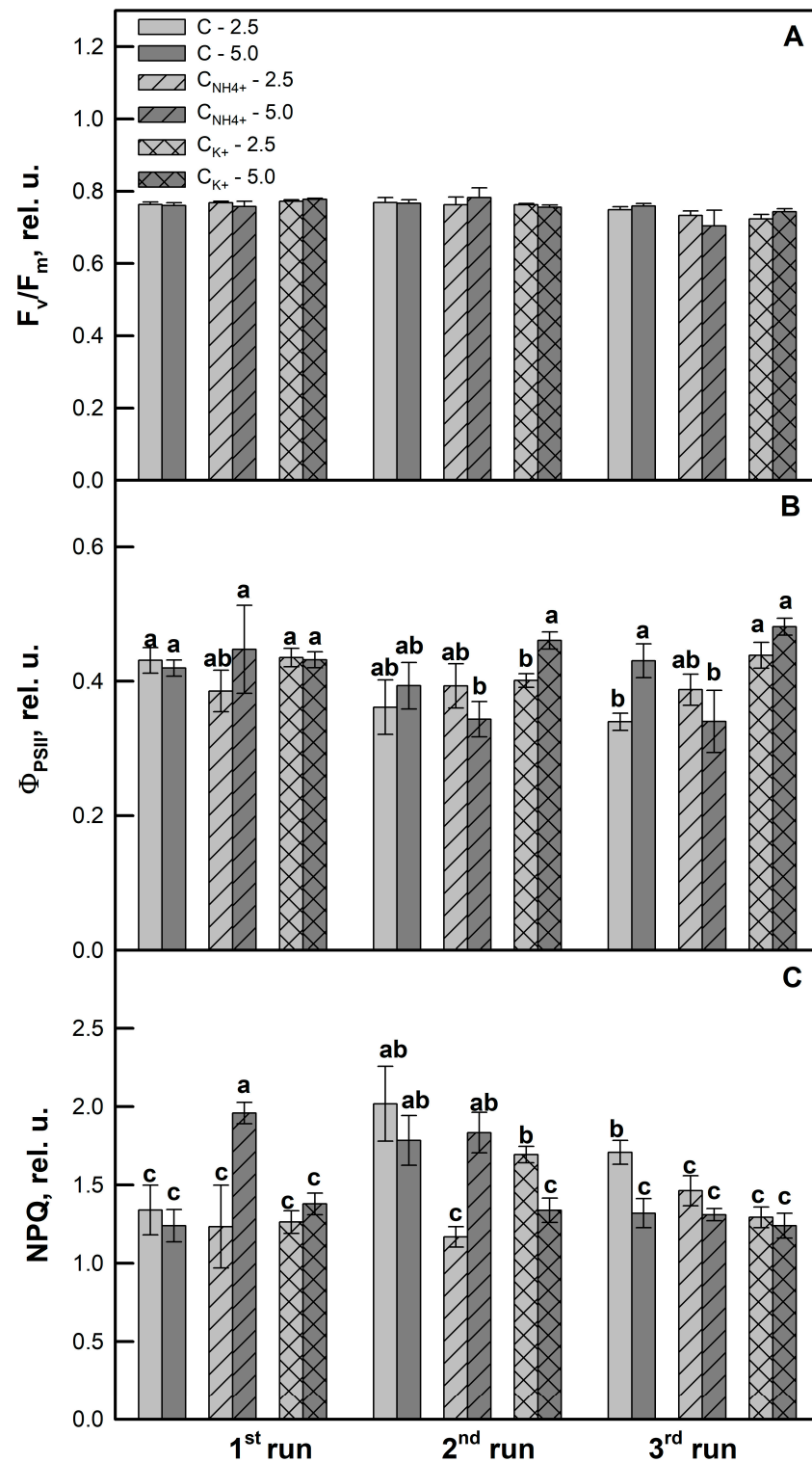


Figure 4. Chlorophyll fluorescence parameters determined on intact leaves of *Raphanus sativus* plants grown on two fractions of non-modified clinoptilolite (C-2.5 and C-5.0), and clinoptilolite modified with ion exchange with ammonium (C_{NH4+}-2.5 and C_{NH4+}-5.0) and potassium (C_K-2.5 and C_K-5.0) ions. The 1st run was performed on non-utilized substrates, and 2nd and 3rd runs are complete cultivation cycles on the utilized substrates. Maximum quantum yield of photosystem II in the dark (F_v/F_m, (A)), quantum efficiency of PSII photochemistry in the light (Φ_{PSII}, (B)) and non-photochemical quenching of Chl *a* fluorescence (NPQ, (C)). Data are means ± SE (n = 6). One-way ANOVA followed by post hoc Tukey’s test were applied, and statistically significant differences are indicated using different letters at p < 0.05.

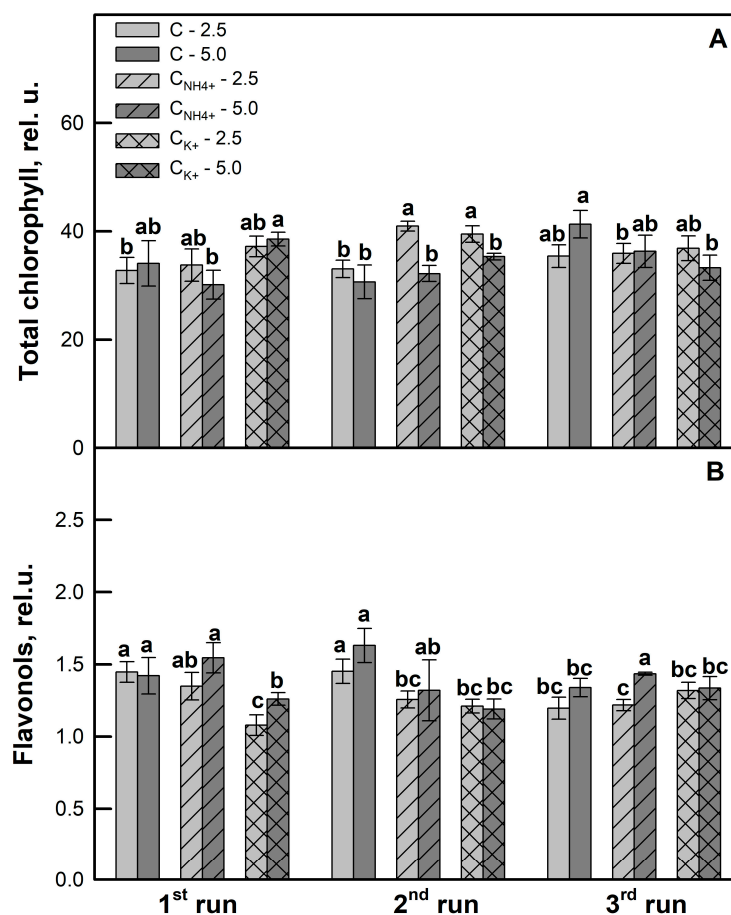


Figure 5. Total chlorophyll (A) and flavonol (B) abundance. Leaf pigment content for *Raphanus sativus* plants grown on two fractions of non-modified clinoptilolite (C-2.5 and C-5.0), and clinoptilolite modified with ion exchange with ammonium (C_{NH4+}-2.5 and C_{NH4+}-5.0) and potassium (C_{K+}-2.5 and C_{K+}-5.0) ions. The 1st run was performed on non-utilized substrates, and 2nd and 3rd runs are complete cultivation cycles on the utilized substrates. Data are means \pm SE (n = 6). One-way ANOVA followed by post hoc Tukey's test were applied, and statistically significant differences are indicated with different letters at $p < 0.05$.

The flavonoid content was lower for C_K-2.5 and C_K-5.0, and it remained lower during the second and third run (Figure 5B). A significant reduction of flavonols was found in C-2.5 plants only after the third run.

3.4. Effects of Clinoptilolite Particle Size and Ion-Exchange Modification on Plant Biochemical Characteristics

Biochemical analyses on the edible roots of radish plants are shown in Figure 6. Data indicate that the reuse of the substrate and the size of particles did not affect the total protein amount. Significant differences among variants were only observed depending on the ion-exchange procedure. In detail, C_K-2.5 (first run) and C_K-5.0 (third run) exhibited a lower protein content ($p \leq 0.05$) than C-2.5 (first run) and C-5.0 (third run) (Figure 6A). We assume that the role of K is essential—being actively involved in the regulation of the balance between reactive oxygen species and antioxidants to adjust protein synthesis and stomatal function—thereby improving the overall photosynthetic performance [36].

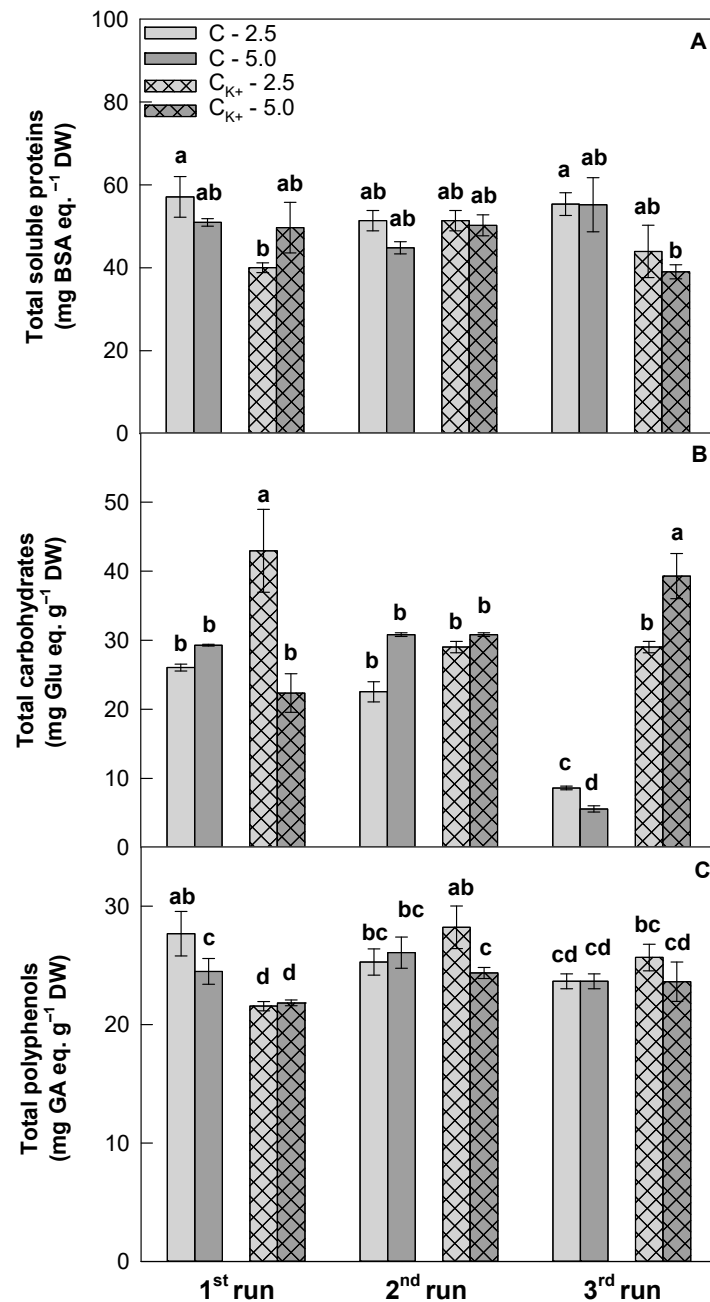


Figure 6. Total soluble proteins (A), carbohydrates (B) and polyphenols (C) in ipogea portions of *Raphanus sativus* plants grown on two fractions of non-modified clinoptilolite (C-2.5 and C-5.0), and clinoptilolite modified by ion exchange with potassium (C_K-2.5 and C_K-5.0) ions. The 1st run was performed on non-utilized substrates, and 2nd and 3rd runs are complete cultivation cycles on the utilized substrates. Results are means ± standard errors of n = 3 measurements. Different letters indicate statistically significant differences among treatments ($p < 0.05$) according to one-way ANOVA analysis.

Generally, the increase in protein content is associated with the high amount of SiO₂ (about 60%) composing the zeolite structure. It boosts the activity of H⁺-ATPase pumps on the plasma membrane, thus enhancing the stability of cellular membranes and controlling the uptake of nutrients [35]. In our case, no significant variation in SiO₂ concentrations was detected during the three growth cycles, regardless of the particle size and ion exchange, and these results may contribute to explaining the reason why we did not observe any changes in the soluble protein content.

The radish root analyses also evidence the presence of large amounts of secondary metabolites, minerals, vitamins, and carbohydrates according to the findings of Mitsui et al. [37]. More specifically, all samples exhibited higher values of total soluble carbohydrates ($p < 0.01$) than those detected in C-2.5 and C-5.0 plants during the third run. We suppose that, rather than particle size, the procedures with KNO_3 have had a substantial effect, as among treatments, the highest values of total carbohydrates ($p < 0.05$) were observed in C_K -2.5 (first run) and C_K -5.0 (third run) (Figure 6B). Rahimi et al. [31] evidenced the presence of higher concentrations of sugars in leaves of perennial ryegrass plants grown on ordinary clinoptilolite zeolite or potassium-enriched clinoptilolite. The sugar translocation toward radish roots is involved in protecting the cellular machinery, absorbing water from the rhizosphere, and improving osmotic adjustment.

Moreover, as observed in beet or carrot crops, also in radishes, the increase in the photosynthetic rate, meristematic growth, water use, and sugar translocation may contribute to enhancing the K uptake and accumulation of nutrients into roots [38].

The radish is a species also recognized for its high medicinal and nutritional value, especially as a fount of polyphenols, or other compounds of therapeutic interest involved in disease prevention and health promotion [39]. In our study, polyphenols showed the highest concentration ($p < 0.05$) in C-2.5 (first run) and C_K -2.5 (second run), exhibiting comparable values (Figure 6C). The lowest ($p < 0.05$) concentrations were detected in both C_K -2.5 and C_K -5.0 samples for the first run.

Our analyses evidenced that the second run promoted the production of polyphenols in radish bulbs, while no remarkable effect related to the ion-exchange procedure or particle size was found. We measured concentrations comparable to or higher than those reported in previous studies [39–41], and hypothesized that some differences may be likely due to several factors influencing polyphenol biosynthesis, including intraspecific diversity, plant breeding, post-harvest handling [42], variety and vegetative stage [43] or cultivation substrate. Ammonium-enriched zeolite (composed of 90–95% clinoptilolite) was indeed reported to increase phenolic contents in strawberry fruits, boosting the production of compounds with antioxidant, cardioprotective and anticarcinogenic activities [34].

4. Conclusions

One of the first steps if humans ever want to settle on the Moon and Mars is to ensure that they will be able to grow their own food to support long-duration missions. A number of studies have been conducted in this direction; however, much research is still required on the challenges of growing crops for space [44].

In the present study, we have explored a number of different analyses in order to understand the correlation between clinoptilolite characteristics (particle size, chemical composition) and plant behavior (seed germination, plant growth, physiological and biochemical traits). It has been found that the ion exchange did not cause any changes in the crystalline structure of clinoptilolite. However, the degree of exchange with potassium ions was greater than with ammonium ions. The extent of replacement with potassium was higher in the bigger fraction (2.5–5.0 mm) compared to the smaller one (0.9–2.5 mm). This observation could be related to the structural positions occupied by potassium which are more accessible for a substrate with a larger particle size. Here, for the first time, we have reported that the ion-exchange process affects the water-holding capacity of clinoptilolite, and this effect depends on the utilized chemical compounds (ammonium nitrate or potassium nitrate). Ammonium exchange increased the water-holding capacity of the zeolite up to 60%, while potassium-exchanged substrates possessed lower water-holding properties (reaching the maximum of 45%). Those effects were not dependent on the particle size of the zeolite.

Small fractions of ammonium- and potassium-ion-exchanged clinoptilolite positively affect seed germination, being the most pronounced during the third run, showing 90% germination in C_{NH_4} -2.5 and C_K -2.5 plants, while the germinability in C-2.5 was 75%. Root crop production was also favored by the small clinoptilolite fractions, especially

in C_K-2.5 plants, being twice higher than in C-2.5 during the first run. However, the utilization of these substrates reduced the accumulation of root crop biomass, which was especially evident during the third run. Photochemical reactions of photosynthesis were neither significantly affected by the ion exchange nor by the particle size. Concerning root crop biochemical traits, no significant variations in protein content were observed, while an important increase in total carbohydrate and polyphenol contents were found in root crop grown on potassium-exchanged clinoptilolite, exhibiting maximum values in C_K-2.5 (first run, 42.9 ± 6.00 mg Glu eq. g⁻¹ DW) and C_K-2.5 bulbs (second run, 28.2 ± 1.79 mg GA eq. g⁻¹ DW), respectively.

Our study provides new insights for better utilization of a widespread material (clinoptilolite) as a substrate for plant growth in space-oriented experiments. Based on the chemical and structural properties of clinoptilolite, it is worth investigating whether this zeolite could influence the response of seeds and consequently plants to other space factors, such as ionizing radiation and altered gravity. Likewise, as the impact of zeolite application varies depending on its nature, availability, application approaches and grain size distribution, further investigations are needed to maximize zeolite implications in terms of sustainability and the reuse of suitable resources at limited costs.

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