



How Mucilaginous Seeds of Different Plant Species Respond to Nonthermal Atmospheric Plasma Treatment

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Abstract

Important representatives of mucilaginous seeds from different plant species, namely amaranth (*Amaranthus hypochondriacus* L.), garden cress (*Lepidium sativum* L.), common flax (*Linum usitatissimum* L.), psyllium (*Plantago ovata* Forssk.), and chia (*Salvia hispanica* L.) were subjected to non-thermal plasma (NTP) generated by diffuse coplanar surface barrier discharge with different exposure times (1, 3, 5, 10, 20, 30 s). Seed water uptake, kinematic viscosity, parameters of seed germination and initial seedling growth were monitored along with chemical and morphological changes on the seed surface. Water absorption increased with increasing plasma exposure time for garden cress, psyllium and chia seeds, but it was greatest for chia seeds. For all seed species, the kinematic viscosity decreased with increasing plasma exposure time. The highest values were found for chia seeds after a treatment for 30 s. Surface analyses did not reveal any chemical and morphological changes of the seed surface. According to a PCA comparison of basic characteristics of germination and initial growth, common flax seeds differ in their reaction to NTP from the other tested plants. On the contrary, chia seeds showed the best water uptake and kinematic viscosity. It was shown that NTP treatment improves the absorption of mucilaginous seeds and does not change the surface and structural properties of the seeds. These mucilaginous seeds can be used as raw seed, whereby NTP accelerates their preparation during soaking.

Keywords Non-thermal plasma · Seed germination · Early growth · Kinematic viscosity · Water uptake · Seed coat anatomy

Introduction

In this work, we have focused on plants that have slimy coverings on the seed surface called mucilages (or exudates). These mucilages are usually polysaccharides (i.e. celluloses, pectins or hemicelluloses), that are formed just after hydration and surround the seed as a gel-like, transparent capsule [1]. Mucilage has different ecological functions: it protects the seeds from desiccation, fixes the seeds in the soil, protects the seeds from predation, influences seed germination and serves as a source of energy for the seeds

[2]. Mucilaginous seeds can have multiple benefits for human health [3–6] and have great potential for use in functional foods [7–9]. Above all, mucilaginous seeds represent a suitable food supplement and a source of important minerals and fiber. Currently, interest in researching plant seeds as vegetarian food for humans is increasing from year to year.

Similar to cross-linked hydrogels, mucilages have the ability to absorb large amounts of water due to their net-like structure and thus retain moisture around the seed [1]. Since mucilages are composed of glycoproteins and polysaccharides, they have a hydrophilic character that supports the high efficiency of water absorption. In our study, we focused on selected seed types, namely amaranth, garden cress, common flax, psyllium and chia seeds. All these seeds have a mucilaginous testa (seed coat) and are considered important food or pharmaceutical sources. We aim to investigate the effects of non-thermal plasma (NTP) on the surface properties, germination and early growth characteristics of these seeds. Furthermore, the investigation of the water uptake capacity influenced by plasma treatment is an important objective of this work, as well as the study of changes in the viscosity of the mucilage.

NTP represents a very reactive environment consisting of photons, electrons, ions, atoms, free radicals and excited or unexcited molecules. The electron energies are high and thanks to them reactive oxygen and nitrogen species (RONS) can be generated in gases containing oxygen and nitrogen. Together with UV radiation, RONS play an important role in the interaction with the different substrates including for example also seeds. NTP is frequently used in agricultural research considering its positive effect on seed germination and ability to accelerate seedling growth in the early stages of germination, resulting in improved seedling vigour [10–15]. Other studies show the formation of active compounds in the sprouting seeds inducing by plasma exposure [16, 17], which increases the nutrient content of the plants and can extend the shelf life of fruits and vegetables in the post-harvest phase [18, 19]. On the other side, NTP full of various active species can negatively influence the viability of various microorganism and fungi and it is also known for its decontaminating effect, which helps to prevent the development of many seed diseases during long storage periods [20–23]. Moreover, an efficient plasma can help remove pesticide residues from plants used during sowing [24, 25]. Due to the low temperature during operation, the NTP is very gentle on sensitive biological material such as seeds, avoiding mechanical damage and affecting only a thin layer of the treated substrate. By selecting the appropriate plasma device from the commonly used plasma sources (plasma jet, gliding arc, corona discharge and/or dielectric barrier discharge) [26] we can minimize treatment times to seconds and simplify the process by operating at atmospheric pressure in ambient air.

So far, the NTP has been used to treat various types of seeds, usually of agriculturally important crops used for massive food production, such as soybean [27], wheat [21], maize seeds [28], rice [29], barley [30], sunflower [31], pea [32] as well as many others as can be found in the review articles [10–15]. The mucilage seeds analysed in our study have only been subjected to plasma treatment in a few works. Common flax treated with radiofrequency plasma in a vacuum medium showed an improvement in wettability and water uptake [33] similar to the study by Dubinov [34], in which the treatment of seeds with dielectric barrier discharge (DBD) plasma shortened water penetration and increased the germination rate. In another study, the DBD type of plasma source was also used for the treatment of garden cress [35]. In addition, chia seeds and the mucilage obtained from them were treated with argon jet plasma, whereby the properties of the mucilage were analyzed in detail [36, 37].

To achieve the desired effect of the plasma to the seeds related to specific goal of the application, it is necessary to find suitable plasma treatment parameters, which of course depend on the type and properties of the seed, the type of plasma source used and the properties of the plasma (its composition, radiation intensity, plasma density, etc.) and the length of plasma treatment time.

The present study is a complex investigation of the influence of NTP generated by diffuse coplanar surface barrier discharge (DCSBD) [38] on the water holding capacity of selected seed species, namely amaranth, garden cress, common flax, psyllium and chia seeds. As a part of this study, the surface properties, germination and early growth characteristics of these seeds as well as the kinematic viscosity changes of the mucilage are analyzed.

Materials and Methods

Plant Material

Seeds/fruits of five investigated plant species, named amaranth (*Amaranthus hypochondriacus* L.), garden cress (*Lepidium sativum* L.), common flax (*Linum usitatissimum* L.), psyllium (*Plantago ovata* Forssk.), chia (*Salvia hispanica* L.), were obtained in organic quality from commercial suppliers in 2022 in the Czech Republic. Only ripe, intact, surface-untreated (unpeeled) and surface correct fruits were used in this experiment. 150 seeds/fruits per one plant species were used for each plasma treatment (different exposure times) and for reference samples. We name investigated seeds/fruits as seeds in this text. All attempts took place during 2022 and at the turn of 2022/2023. The thousand seed weights (WTS) and seed germination rates for each species are shown in Table 1; Fig. 1 brings photos of all tested seeds.

Plasma Treatment

The plasma treatment was conducted using the Diffuse Coplanar Surface Barrier Discharge (DCSBD) (Fig. 2) with previously published experimental set-up suitable for the treatment of seeds [39]. This system generates a nonthermal plasma at atmospheric pressure due to the use of alternating voltage and the presence of a dielectric barrier in its electrode system, which consists of 16 pairs of parallel strip-line silver electrodes embedded below the surface of a ceramic plate (Al_2O_3 , 96% purity). At ~ 15 kHz sinusoidal high voltage with an amplitude of up to ~ 20 kV peak-to-peak, supplied by a HV generator VF 700 (Lifetech Ltd., Brno, Czech Republic), the DCSBD generates a thin (~ 0.3 mm), macroscopically homogeneous plasma layer on an active area of $(20 \times 8) \text{ cm}^2$ [39]. The DCSBD plasma generated in air produces a multitude of reactive oxygen and nitrogen species [40], which play a significant role in affecting the seed properties. The high surface power density of the DCSBD plasma (2.5 W/cm^2) ensures that the treatment is effective after a very short exposure times. The continuous operation of the DCSBD is enabled by an effective cooling and insulating oil system between the electrode strips, which also prevents overheating of the ceramic surface. More detailed characteristics of the DCSBD plasma source have been described in previous works [41, 42]. Plasma treatment of the seeds was carried out in ambient air at exposure times of 1, 3, 5, 10, 20, and 30 s. The exposure times were chosen based on our previous experience with seed treatment of different types and sizes [21, 39]. The optimal treatment time using our DCSBD plasma

Table 1 Basic characteristics of studied seed species

Basic characteristics/Common name	Amaranth	Garden cress	Common flax	Psyllium	Chia
Latin name	<i>Amaranthus hypochondriacus</i> L.	<i>Lepidium sativum</i> L.	<i>Linum usitatissimum</i> L.	<i>Plantago ovata</i> Forssk.	<i>Salvia hispanica</i> L.
Family	Amaranthaceae	Brassicaceae	Linaceae	Plantaginaceae	Lamiaceae
Seed/fruit	fruit: achene	seed	seed	seed	fruit: nutlet
WTS (g)	0.729	2.351	8.302	1.815	1.132
Seed germination (%)	66.0	98.3	78.7	88.7	64.7

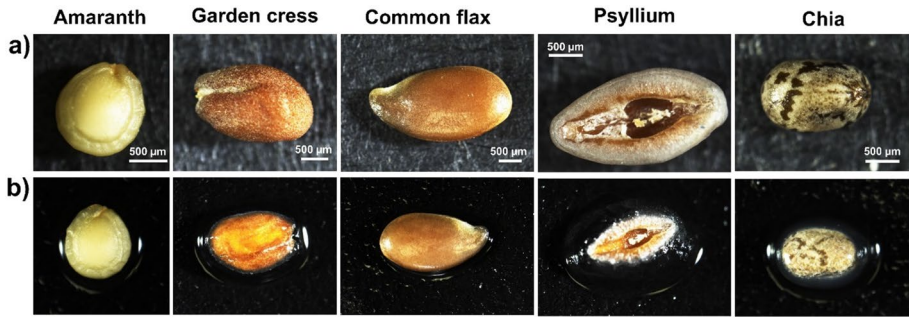


Fig. 1 Photos of all tested seeds **a** in dry state and **b** with visible mucilage around the seed

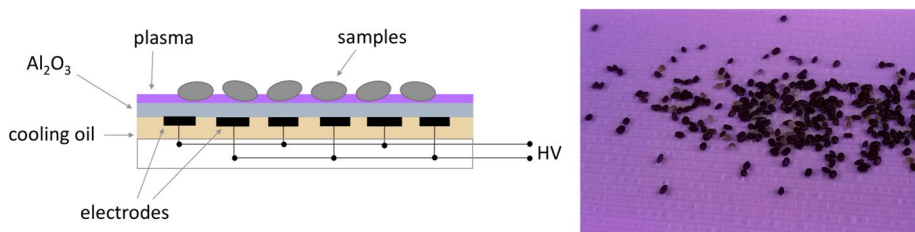


Fig. 2 Scheme of DCSBD discharge configuration with strip-line electrodes situated on the bottom of the ceramic plates (left), the photo of chia seeds in DCSBD plasma area during treatment (right)

source was 10–30 s for most of the smaller seeds examined. As mentioned in our previously published articles, the length of the plasma treatment is important and depends on the type of seed, its surface properties (e.g. hardness) and the location of the seed germ relative to the surface and of course on the properties of the plasma source used. At the same time, we wanted to keep the germ in the seed alive and capable of germinating or form mucilage on the surface. The input power was maintained at 400 W using a commercial energy consumption meter FK technics 3362. To ensure homogeneous treatment of the seeds in the plasma layer, the seeds have been moved in the plasma layer using a silicone brush.

Seed Water Uptake

Dry seed samples ($m_o \sim 1$ g) from each plant and each plasma treatment were randomly selected and then soaked in distilled water (50 mL) for 5–35 min and then weighed (m) with laboratory scales (Nahita-blue, series 5133, precision balance 0.001 g). Before each measurement, the seed samples were placed on sterilized paper to remove the excess of water. The measurement was performed every 5 min for each treated sample separately (including the control sample) in three repetitions. Seed water uptake was understood as a relative increase of weight and the following equation was used for calculation [43]:

$$\text{Seed water uptake} = (m - m_o) / m_o$$

where m_0 is the initial total mass of the seeds and m is the total mass of seed samples imbibed for each measurement time. Seed water uptake (average values) was visualized for individual plant species as a function of time (0–35 min).

Kinematic Viscosity

Kinematic viscosity was measured in laboratory conditions, all measurement steps were carried out at an air temperature of 24–25 °C. The randomly selected seed sample (1 g) from each plant and each plasma treatment was transferred to a 100 mL glass container with a cap, the seeds were moistened with 1 mL of 96% ethanol dehydration, then 25 mL of distilled water was added and the container was closed. The amount of ethanol and distilled water were added based on previous trial tests. The glass container was shaken after 10 min for 1 h (1000 RPM) by multi-speed vortex MSV-3500 (Biosan, Latvia). The seeds adhering to the glass surface were incorporated by a slight vertical circular motion of the container, after which the container was allowed to stand for 3 h. After this rest, most of the liquid was bound to the seeds, the excess water was poured off. Kinematic viscosities were determined with the Ubbelohde viscometer (SI Analytics GmbH, Germany) under a constant temperature at 25 °C with an error rate of 5% within the measurement. Kinematic viscosity value (mm^2/s) was measured three times for each plant species and for these treatments: 1, 5, 10, and 30 s.

ATR-FTIR Spectroscopy

Chemical changes on the surface of the mucilaginous seeds were investigated by ATR-FTIR spectroscopy using same measurement method as in our previous study [39]. Briefly, the ATR-FTIR spectra were measured using the Bruker Vector 22 spectrometer (Bruker, Germany) with additional accessory Pike MIRacle™ (with diamond crystal). Three different samples of each seed type from one batch were measured after plasma treatment (used 30 s plasma exposure time) and compared with reference spectra, which were also measured three times. Seeds after the plasma treatment were moved from the DCSBD ceramic plate by the paper shovel into the glass beaker. Then the seeds were taken by the small metal spoon and placed on the crystal with special ATR sample holder to avoid the moving of the seeds. The spectra were recorded with a resolution of 2 cm^{-1} in the range $4000\text{--}600\text{ cm}^{-1}$ with 70 scans for each sample and analysed with OMNIC software (version 8.0, Thermo scientific) using baseline correction (linear algorithm) and smoothing (Savitzky–Golay algorithm) of the spectral lines.

SEM Analyses

The micromorphological characteristics of untreated and plasma-treated seeds were evaluated by scanning electron microscopy (SEM), using a Tescan Lyra 3 SEM microscope (Tescan, CZ) with an accelerating voltage of 5 kV in regime of secondary electrons and with magnifications of 5–25 kx depending on the type of seed, its size and surface morphology. The samples subjected to this analysis were treated with plasma for 30 s, which was the longest treatment time, to register possibly plasma damage of surface.

Seed Germination and Early Growth

For these analyses we adapted the previously used protocol and modified it according to the specific requirements for this study [39, 44] and it reads as follows:

Thirty seeds of each plant species, one layer of KA0/110 filter paper and 6 mL of distilled water were used per one plastic Petri dish of 120 mm in diameter. All experiments were repeated 5x in parallel (5 Petri dishes for one treatment); so, each treatment and control variant contained 150 seeds for one plant species. All Petri dishes were kept in a growing box at a temperature of 25 °C and in the dark for the whole day (24 h/no light). The growth cultivation lasted 6 days. The 3rd and 6th day of data measurements were established according to data from previous short germination test.

The number of germinating seeds and the root length (LR3, LR6) were measured on the 3rd and 6th day of seed cultivation. Data on shoot length (LS), fresh weight of shoot (WFS), and fresh weight of root (WFR) were measured on the 6th day. Then, shoots and roots were dried separately at 70 °C for 24 h and the weight of the dried shoots (WDS) and roots (WDR) was determined.

Seed germination (G3, G6) was calculated as a percentage proportion between the number of germinating seeds and the number of seeds used in the germination test. Germination rate (GR) and germination index (GI) were calculated according to recently published work [45]. Seedling length (L) was calculated as the sum of root length (LR6) and shoot length (LS) for each seedling; the fresh (WF) and dried weight of seedling (WD) were calculated from WDS and WDR.

Root/shoot ratios were calculated from the length (R/S_L), fresh weight (R/S_FW) and from dry weight (R/S_DW) of root and shoot. Three seedling vitality indexes were determined as the product of seed germination and seedling length (SVI_I), seed germination and seedling fresh weight (SVI_II), and seed germination and seedling dry weight (SVI_III) [45].

All the above characteristics describing seed germination and initial seedling growth were determined for all studied plant species and for all non-thermal plasma treatment times (and control samples).

Data Analyses

The experimental measurements were repeated five times and data analysis was performed similarly as in our previously published paper [39, 44]. The dataset was first logarithmically transformed ($y = \log(x)$) to normalize the data. All obtained characteristics from investigated plants were considered as explanatory (active) and treatment with non-thermal plasma at different times as explanatory (supplementary). Statistical analyses were calculated using the R project (version 4.3.1), the ggplot2, ComplexHeatmap, corrplot, and FactoMineR packages. All calculations were performed at the significance level of 0.05. Statistical analyzes were calculated for each plant individually.

T-tests were used in seed water uptake to evaluate differences between treated seeds and control set.

Factorial analyses were used to evaluate the influence of the plant species (factor Plant), the duration of the NTP treatment (factor Time) and their combination (factor Plant*Time) in the kinematic viscosity.

One-way analysis of variance (ANOVA) was used to evaluate the influence of the NTP treatments on the seed and seedling characteristics. The detailed testing of experimental variances was performed using post hoc multiple comparisons (PHC) by Tukey's HSD test. Comparisons between the control and individual NTP treatments (both for germination and initial growth and for the amount of mucilage) are presented [39].

A principal component analysis (PCA) was used to evaluate the relationship between the responses of all tested plant seeds to the NTP treatments (0, 1, 3, 5, 10, 20, 30 s). These basic characteristics of seed germination and early seedling growth were used: G3, G6, LR3, LR6, WFS, WFR, WDS, and WDR.

Results

Seed Water Uptake

The generated seed water uptake curves had different shapes for different types of tested seeds (Fig. 3). The classic course, i.e. an exponential increase and subsequent flattening of the curves, was visible in chia and garden cress plants. In common flax and psyllium species, seed water uptake curves were very steep and linear in the first stage of measurement (up to 5 min). For amaranth seeds, the water uptake curves were almost linear throughout the time (0–35 min). Similarly for the curves for psyllium in the interval 5–35 min. It is likely that the amaranth and psyllium seeds were not sufficiently soaked in water at the end of the experiment.

The water uptake was found to increase with increasing plasma treatment time for garden cress, psyllium, and chia seeds (Fig. 3). The highest water uptake values were recorded for all NTP-treated chia seeds, with a demonstrable difference ($p < 0.05$) compared to the control seeds. No significant differences between treated seeds and control sets were noted in other tested species. The lowest water uptake values were recorded in common flax seeds.

Kinematic Viscosity

The factorial analysis found, that both single factors (Plant and Time) were important, because significant differences were observed in the kinematic viscosity ($p < 0.05$, Table 2). This means that the type of plant and the treatment time used play a major role in the kinematic viscosity. On the other hand, the factor combination (Plant*Time) was the weakest, with no significant difference found (Table 2). The combination of both factors canceled out the significance of these individual factors.

An overview of the measured kinematic viscosity values can be found in Table 3. No significant difference was found between treatments within each seed type. Nevertheless, there are differences in values, and therefore we can talk about possible trends. When comparing different NTP treatment times within individual plants, trends are visible where higher times show lower kinematic viscosity. These differences are not at the level of significance within individual plants (Table 3). The highest kinematic viscosity values were recorded for chia seeds ($1.58 \text{ mm}^2/\text{s}$) and the lowest for amaranth seeds ($1.05 \text{ mm}^2/\text{s}$), which were not treated (0 s, control sets). The largest difference in kinematic viscosity between control and 30 with NTP treatment was noted for flax (13%) and the smallest difference for chia (5%).

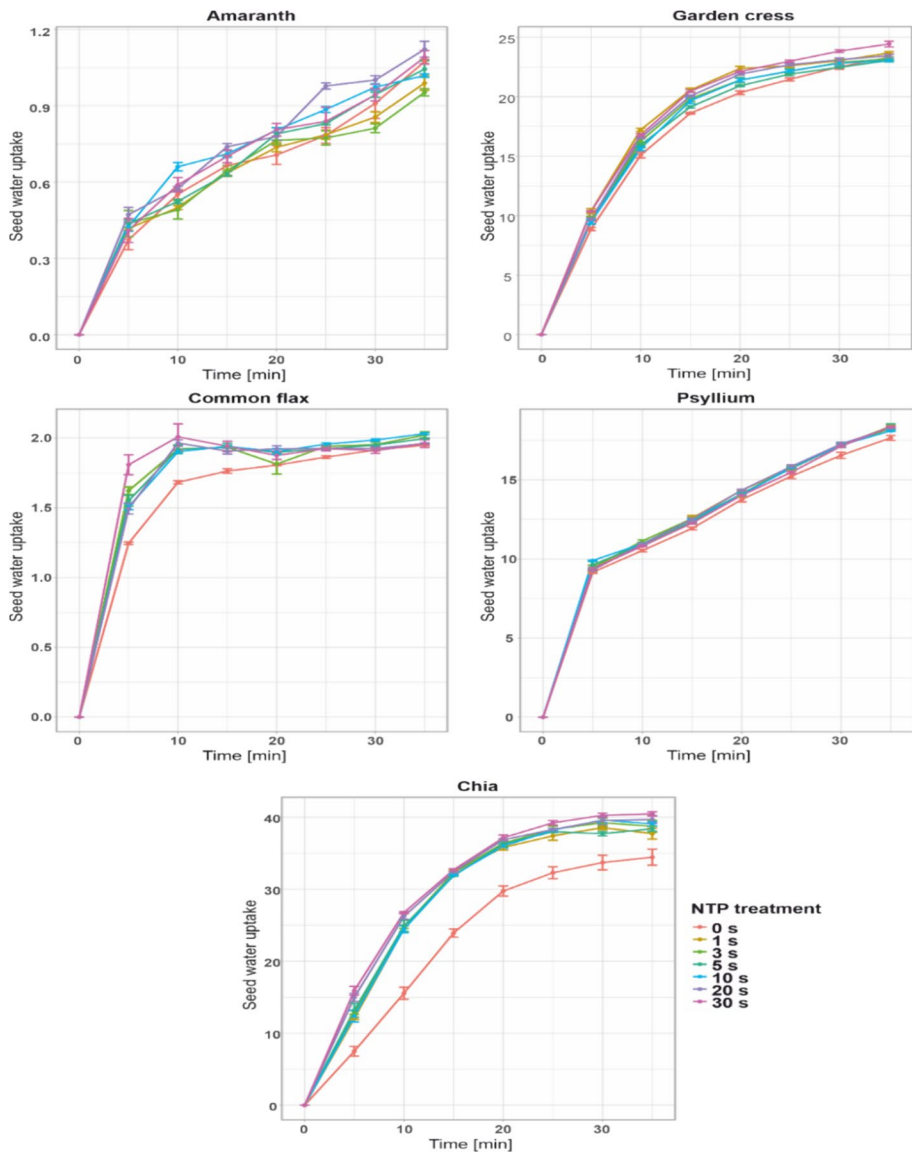


Fig. 3 Effect of DCSBD plasma treatment duration on seed water uptake during 0–35 min in all tested plants. Significant differences (t-test, $p < 0.05$) were found only in chia seeds, between all treated seeds (1–30 s) and control set (0 s)

Seed Surface Characterization

The ATR-FTIR spectra displayed in Fig. 4b–f show the spectral lines before and after plasma treatment for all analyzed seeds. We measured FTIR spectra of seeds treated for 30 s. When looking at the spectra pairs in Fig. 4b–f, we could not detect any new peaks caused by the plasma treatment. There are only slight differences between the individual

Table 2 Results of the factor analysis, univariate significance tests for kinematic viscosity are given

Treatment	DF	Mean square	F	<i>p</i>
Replication	1	90.70	12309.18	0.000
Plant	4	0.44	60.28	0.000
Time	3	0.03	4.75	0.006
Plant*Time	12	0.00	0.15	0.999
Error	40	0.01	–	–

Significant differences are highlighted with a bolt ($p < 0.05$)

Table 3 Kinematic viscosity in tested plant seeds after application of DCSBD plasma

Plant	Time (s)	Mean	SE	PHC
Amaranth	0	1.05	0.07	ns
	5	1.04	0.03	ns
	10	1.01	0.04	ns
	30	0.98	0.02	ns
Garden cress	0	1.19	0.03	ns
	5	1.14	0.02	ns
	10	1.09	0.02	ns
	30	1.07	0.02	ns
Common flax	0	1.27	0.03	ns
	5	1.21	0.03	ns
	10	1.20	0.02	ns
	30 s	1.10	0.09	ns
Psyllium	0	1.33	0.05	ns
	5	1.30	0.01	ns
	10	1.27	0.04	ns
	30	1.20	0.04	ns
Chia	0	1.58	0.04	ns
	5	1.53	0.04	ns
	10	1.51	0.03	ns
	30	1.50	0.14	ns

Treatment duration (time), average kinematic viscosity values (mean, $\text{mm}^2 \text{s}^{-1}$), standard error (SE), post hoc comparison test (PHC), non-significant (ns), details see in method

seed types, as can be seen in Fig. 4a. The intensity and shape of the $3000\text{--}3600 \text{ cm}^{-1}$ band, which is assigned to the OH/-NH stretching vibrations, is highest for common flax and lowest for psyllium. The symmetrical and asymmetrical C-H stretching vibrations of the CH_3 , CH_2 and CH groups in the range of $2800\text{--}3000 \text{ cm}^{-1}$, which are normally assigned to lipids in seed samples, are also differently pronounced in the corresponding seeds. In the spectra of psyllium and common flax, these peaks have a very low intensity, while the intensity in the spectra of the other seeds is relatively high. The spectral range of $1200\text{--}1800 \text{ cm}^{-1}$ describes the presence of proteins in biological samples with peaks assigned to the amide I, II and III bands, which differ moderately in the monitored

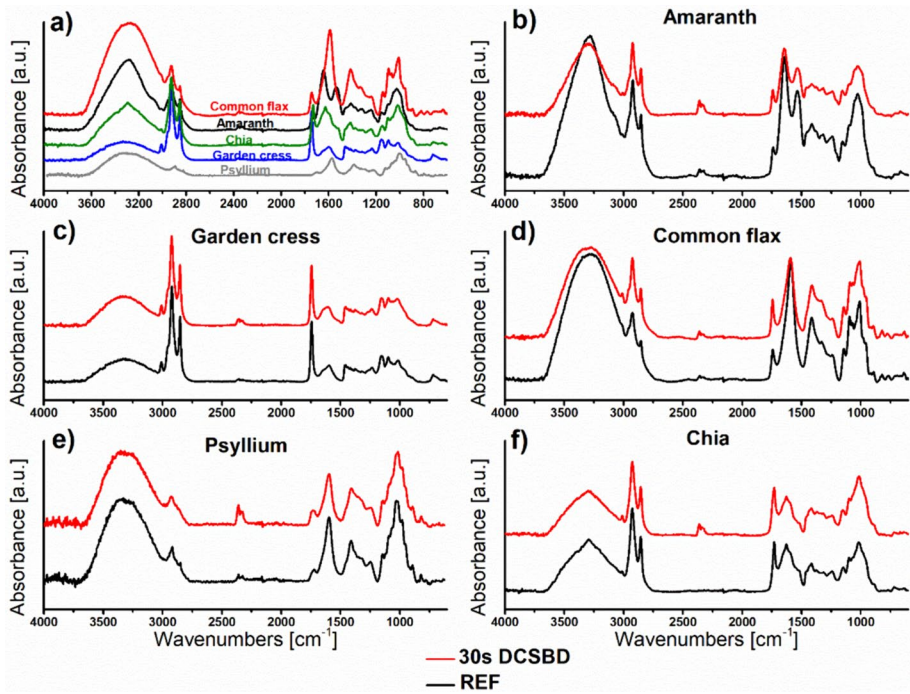


Fig. 4 ATR-FTIR spectra of **a** all seed types and compared spectra before and after DCSBD plasma treatment using the plasma exposure time 30 s of **b** amaranth, **c** garden cress, **d** common flax, **e** psyllium and **f** chia seeds

seeds. The characteristic peaks for polysaccharides in the $800\text{--}1200\text{ cm}^{-1}$ range also show slight differences between the seed types.

The SEM secondary electron images depicted in Fig. 5 show negligible changes in the surface morphology of the seeds after treatment with plasma at an exposure time of 30 s. For amaranth, common flax, garden cress and psyllium, we found that the small particles

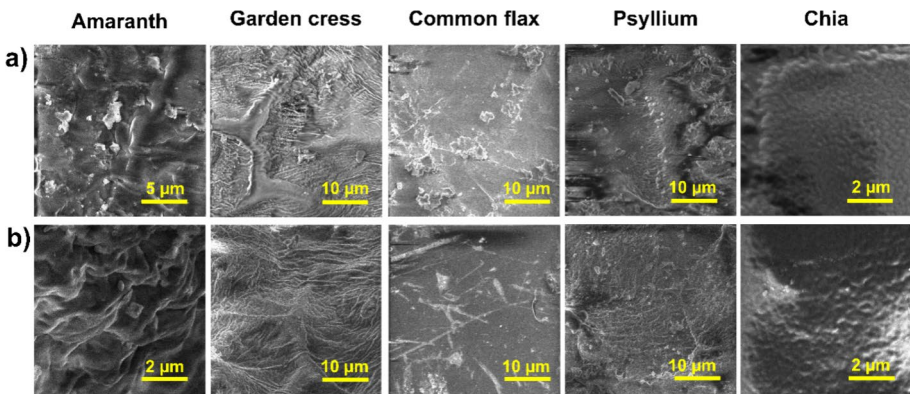


Fig. 5 SEM images of all seed types **a** before and **b** after DCSBD plasma treatment using the plasma exposure time of 30 s observed at the relevant magnifications for every seed type

(maybe dust, or unknown contamination) on the seed surface were removed. Grooves and scratches were visible on the surface of the common flax of the reference sample, probably originating from the processing of the seeds. After plasma treatment, they became more pronounced, which could be explained by plasma cleaning of the surface.

Seed Germination and Early Growth

All the obtained results on the observed characteristics from germination and initial growth are summarized in the appendix. The values of the measured parameters were higher or did not significantly decrease with NTP treatment times of 1–5 s. The times of 10 s and 20 s generally showed a decrease in values compared to the control, and the time of 30 s was fatal for the seeds.

From the germination tests (Appendix), seed germination (last day of test) was higher than the control at the following treatment times: amaranth 3 s, chia 3 ss, common flax 5 s, psyllium 5 s. Garden cress seeds showed no improvement, it germinated at all treatment times. It can be stated that the 30 s treatment was unsuitable for all types of seeds, except common flax seeds, because the optimal treatment time was exceeded. Garden cress seeds did not germinate even after treatment for 20 s (Appendix). The significant differences between untreated and treated seeds were generally observed at longer treatment times, and all these measured values were significantly lower than in the control group. No positive trends in these measured characteristics were noted. Due to the large number of germination and initial growth characteristics measured, the results are analyzed in the Discussion chapter.

According to a PCA comparison of basic characteristics (seed germination, seedling length and weight), common flax seeds differ in their reaction to NTP from the other tested plants (Fig. 6).

Discussion

Mucilaginous seeds are able to absorb large amounts of water because they engage their surface mucilage system. Our experiment suggested that NTP affects water uptake capacity. This ability of the treated seeds is particularly strong for chia, common flax and garden cress seeds compared to the control (0 s) (Fig. 3). On the contrary, the seeds of the amaranth and psyllium species do not show the ability of balanced water uptake (Fig. 3).

These results correspond to the ranking of seed types according to the number of significant differences in the observed characteristics of seeds and seedlings (Fig. 7). According to this review, garden cress, chia and common flax seeds tolerate the NTP treatment relatively well because the sums are low (13, 14 and 17, respectively). Flax is quite different from other plants because its seeds were able to germinate and grow even after high doses of NTP (Fig. 7). Conversely, psyllium and amaranth seeds have these sums high (26 and 34, respectively) and are therefore very sensitive to NTP treatment (Fig. 7). This fact can be attributed to the different nature of the seed coat, the different seed size and the position of the embryo in the seed. Amaranth seeds are the smallest of the seeds tested (Fig. 1) and the embryo is located on the periphery of the seed forming the ringlet surrounding the perisperm, similar to *A. pumilus* [46]. This could indicate that they are the most sensitive to plasma treatment compared to other seed types. The same applies to the “naked embryo” of the psyllium seeds and the very “soft” seed coat [47], so that a lower tolerance of the

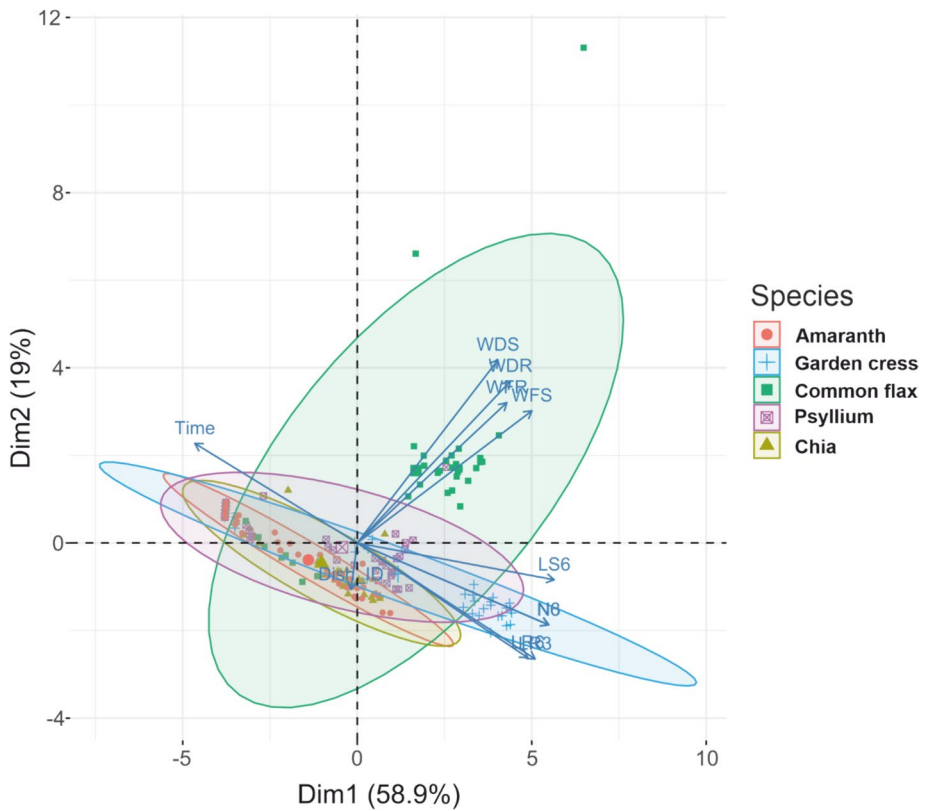


Fig. 6 PCA biplot comparison among all tested plant. The basal characteristics of seed germination and initial seedling growth were used

NTP treatment is conceivable. The other seed types (chia, common flax, garden cress) have harder seed coat that sufficiently protects the embryo located in the middle of the seed, and their size indicates that they tolerate NTP treatment better under the same plasma exposure conditions.

Similarly, the results of germination and growth parameters also reflect the above-mentioned influence of the nature of the seed species, as they differ in their seed coat, its hardness, and the location of the embryo in relation to the surface. The efficiency depends on the treatment time, considering that the plasma source used (DCSBD) generates very volume energy-dense plasma with a high concentration of reactive particles and the presence of UV radiation. In our plasma source, the plasma layer is very thin (approx. 0.3 mm) therefore, larger seeds are partially in the contact with plasma and treatment of the whole seed is ensured by moving on the surface of the discharge. Smaller seeds (chia, amaranth, psyllium,) have a larger part of surface in contact with the plasma, so a shorter treatment time is sufficient. As shown by previous investigations of the effect of plasma DCSBD treatment on various seeds, e.g. peas [48, 49], wheat [21], maize [28], soybean [27], optimal treatment times are between 20 and 60 s, depending on the size of the seeds.

As it follows from the obtained results of germination tests (Appendix) we can determine the optimal plasma treatment times when germination is higher than control. For

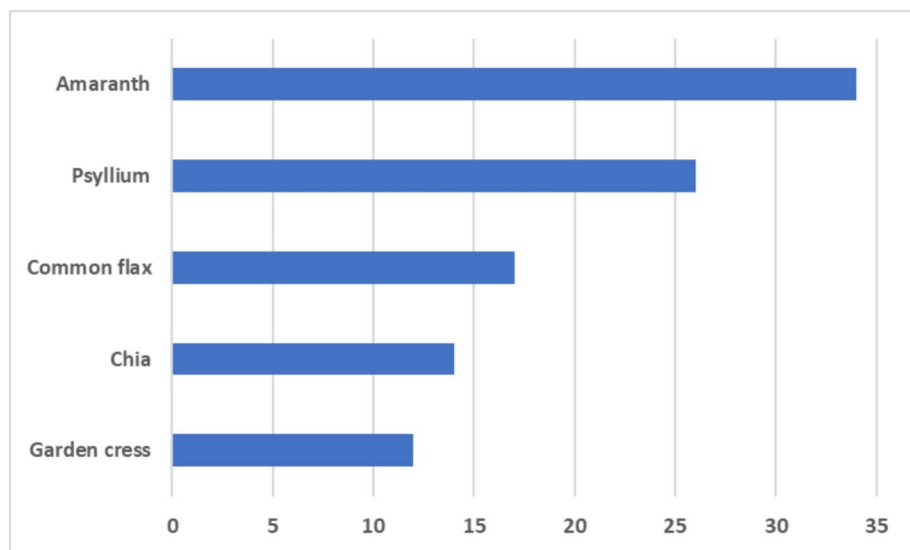


Fig. 7 Summary of the identified significant differences in the characteristics associated with the seed germination and early seedling growth after DCSBD plasma treatments (see Appendixes)

amaranth, a suitable treatment time are probably 3–5 s, for amaranth 3 s, for chia 3 s, for common flax 5 s, and for psyllium 5 s. Garden cress seeds show no improvement, which may be due to the fact that they are intended for consumption and their natural germination rate is low. The higher DCSBD plasma treatment times are not suitable for this type of seed as they reduce germination and growth. We can conclude that adjusted range of plasma exposure time exceeded the optimal time for studied seed species considering their size. The parameters of seed germination and initial plant growth are also influenced by the method and duration of seed storage. Improper storage reduces the vitality of the seeds [45]. The seeds in this experiment come from commercial sales, so we know nothing about their previous storage. Therefore, this important phenomenon was not considered and discussed in this study.

Common flax seeds were the only plants tested that were able to germinate and grow even at higher DCSBD treatment times. Dubinov et al. reported that seeds of the related species Altaic flax (*Linum altaicum*) germinate and grow best with lower times of high-power UV-radiation plasma treatment [34]. However, the improved germinated was supported by the cracked spots in the seed coat caused by plasma exposure. On the contrary, plasma generated by DCSBD is very gentle and does not cause any disruption in the seed coat (Fig. 5).

Differences between seed kinematic viscosity were noted at the level of plant species and NTP treatment time used, and kinematic viscosity decreased in all tested plants with increasing plasma treatment time (Tables 2 and 3). Kinematic viscosity did not correspond to the ability of seeds to absorb water. This is most likely due to the ionic origin of the seed coats that cause seed mucilage. For example, chia seeds imbibed the highest portion of water; however, the decline of kinematic viscosity was the lower from all studied seeds (5%). Possible explanation of this phenomena is that each of the tested plants belongs to a different taxonomic group (Table 1).

Overall, relatively little is known about the interaction of mucilaginous seeds with NTPs. Only chia seeds were exposed to NTP generated by atmospheric pressure cold plasma jet [36, 37]. These studies indicate that all lyophilized mucilages extracted from chia seeds were found to exhibit pseudo-plastic flow behavior and NTP treatment increased the dynamic viscosity of the mucilages. Due to the action of reactive species formed in the plasma generated in noble gases such as argon in this study, the polysaccharide structure of the mucilage was crosslinked, resulting in a more stable network-like structure, which, however, can absorb less water and thus increase the viscosity. On the other hand, the plasma generated by DCSBD in the ambient air has opposite effect to crosslinking and usually causes oxidation of the substrate and can break the chemical bonds. This predominates also in our study, where the mucilage of all seed species was oxidized under the plasma treatment which increases the hydrophilicity along with the better water imbibition for particular seed species as was shown in Fig. 3. Moreover, the bonds breakage could lead to the degradation of the long polysaccharide chains forming mucilages, which can be a reason for viscosity decreasing. Our results showed that chia seeds had the highest values of kinematic viscosity ($1.58 \text{ mm}^2\text{s}^{-1}$) of all the seeds tested and we observed the smallest differences in viscosities between control and plasma treated seeds (5%) (Table 3).

Chia (*Salvia hispanica*) seed belong to the botanical genus *Salvia*. Seeds of some related species have been treated with NTP to break seed dormancy [50] or to study growth potential under stresses [51]. Ghodsimaab et al. worked with dormant seeds of *S. leriifolia*, which are high in fat and protein and have hard seed coats [50]. They observed significant increase in germination percentage and germination rate for plasma treated seed by low-pressure glow discharge plasma at 240 s and water priming. Although, their plasma generated in the air consisted of high density of reactive oxygen and nitrogen species, due to using low pressure plasma source, it necessary to expose the seed to plasma for minutes (240 s = 4 min) in comparison to our DCSBD plasma source, where only few seconds are enough. Alawamleh et al. tested the seeds of the medicinal plant *S. officinalis* (sage) on stress caused by cadmium toxicity [51]. They investigated the independent as well as synergic effect of selenic nanoparticles and NTP to modulate soil cadmium toxicity, with the aim reducing the negative effect of cadmium on sage seed germination. Similar to the previous study, the authors used low-pressure plasma generated by radiofrequency discharge in the vacuum chamber, hence the high plasma exposure time (90 s) was required to achieve the positive effect of plasma. However, selenium NPs and NTP proved to be very beneficial in modulating Cd stress to enhance the photosynthesis rate, and further improve plant growth.

Although the seeds were exposed to the plasma for the relatively long treatment time 30 s which was fatal for all seed types, no changes in the chemical composition could be detected in the FTIR spectra (Fig. 4). Similarly, the SEM images (Fig. 5) did not reveal any significant differences on the surface of the seed coat after plasma treatment. We found that small undefined particles, which could be dust, were removed. Since plasma is also known for its cleaning effect, these impurities were removed by active plasma particles, or the dust was attracted to the ceramic by the electrostatic forces during plasma operation, thus cleaned the seeds.

Regarding the elucidation of the interaction of plasma with seeds and the key role of individual particles, this is not yet fully understood despite extensive research in this area, including ours. This is also due to the fact that it is multidisciplinary research, where cooperate biologists and physicists. In an effort to get a more detailed explanation of the interaction mechanism, we carried out a complex analysis of the composition and properties of the plasma generated by the DCSBD source using the OES and FTIR methods in gases

nitrogen, ambient air, and oxygen at atmospheric pressure [40]. The most radiant system observed in the ambient air and nitrogen plasma is the second positive nitrogen system, which most closely corresponds to the UVA region of 315–400 nm. FTIR measurement showed the presence of RONS (mainly O_3 , NO_2 , N_2O , NO , HNO_2) in the plasma, which could play a significant role in the process of plasma interaction with the seeds. We can, therefore, assume that the positive effect of plasma on the seed is caused not only by individual components of plasma, but also by their synergistic effect, while the ratio of individual active particles as well as the time of plasma treatment or the characteristics of the seeds are important.

Conclusion

In the present study we examined the effect of non-thermal plasma generated at the ambient pressure by DCSBD plasma source on selected seed species characterized by mucilage covering their coat (amaranth, garden cress, common flax, psyllium and chia seeds). Water uptake increased with increasing time of NTP treatment for garden cress, psyllium and chia seeds; the highest water intake was recorded for chia seeds. Trends found in kinematic viscosity point out that higher times indicate lower kinematic viscosity (not significant). The highest values of kinematic viscosity were recorded for chia seeds ($1.58 \text{ mm}^2/\text{s}$), the lowest for amaranth seeds ($1.05 \text{ mm}^2/\text{s}$); the largest difference between control and 30 s plasma treated seed was noted in common flax (13%) and the smallest difference in chia (5%). Decreasing viscosity can be attributed to oxidizing effect of DCSBD plasma generated in the ambient air that could induce the breakage of the mucilage structure into smaller polysaccharide chains. Considering the germination parameters of seeds after plasma treatment, achieved results indicate that selected plasma exposure times exceeded the optimal time for studied seed species. For amaranth, a suitable treatment time when the germination was higher than the control is 3 s, for chia 3 s, for common flax 5 s, for psyllium 5 s. Garden cress seeds show no improvement. Seeds showed different sensitivity to plasma treatment caused probably by different seed size, the nature of the seed coat and placement of the embryo in the seed. Amaranth seeds were the most sensitive to plasma exposure due to very small size and embryo located on the seed coat. Similarly, the plasma could have more destructive effect on psyllium seeds due to the naked embryo and soft seed coat in comparison to harder seed coat of flax, garden cress and chia seeds. We can conclude that an adjusted range of plasma exposure time for individual type of mucilage seeds can lead to the optimization of the desired effects of plasma on kinematic viscosity, water uptake, seed germination and early growth.

Appendix: Overview of Measured Characteristics on Germination and Initial Growth for all Monitored Plant Species

Plants	Treatment time (s)	Germination on the 3rd day (%)			Germination on the 6th day (%)			Germination rate (%)			Germination index		
		Mean	SE	PHC	Mean	SE	PHC	Mean	SE	PHC	Mean	SE	PHC
Amaranth	0	64.0	3.4	–	66.0	3.4	–	97.0	1.9	–	9.7	0.5	–
	1	60.0	5.7	0.980	66.0	3.9	1.000	90.2	4.0	0.997	9.3	0.8	0.997
	3	75.3	4.6	0.258	76.7	4.4	0.242	98.2	1.1	1.000	11.4	0.7	0.222
	5	54.7	2.0	0.476	58.0	2.7	0.566	94.7	3.8	1.000	8.4	0.3	0.468
	10	26.0	3.6	0.000	28.7	3.7	0.000	91.9	7.5	0.999	4.0	0.5	0.000
	20	0.7	0.7	0.000	3.3	1.8	0.000	0.0	0.0	0.000	0.2	0.1	0.000
	30	0.0	0.0	–	–	–	–	–	–	–	–	–	–
Garden cress	0	98.7	1.3	–	98.3	1.3	–	100.3	0.3	–	14.8	0.2	–
	1	97.3	1.6	0.996	97.3	1.6	0.772	100.0	0.0	1.000	14.6	0.2	0.932
	3	96.7	1.8	0.968	96.7	1.8	0.800	100.0	0.0	1.000	14.5	0.3	0.963
	5	98.0	1.3	1.000	98.0	1.3	0.743	100.0	0.0	1.000	14.7	0.2	0.887
	10	64.7	2.7	0.000	67.5	1.9	0.489	95.8	3.1	0.000	9.8	0.3	0.000
	20	0.0	0.0	–	–	–	–	–	–	–	–	–	–
	30	0.0	0.0	–	–	–	–	–	–	–	–	–	–
Common flax	0	74.0	3.2	–	78.7	3.7	–	94.2	1.6	–	11.3	0.5	–
	1	69.3	2.5	0.965	72.0	2.3	0.881	96.3	1.7	0.999	10.5	0.4	0.933
	3	73.3	4.6	1.000	78.7	3.9	1.000	93.2	3.6	1.000	11.3	0.6	1.000
	5	77.3	3.6	0.994	83.3	3.8	0.977	92.9	2.3	1.000	11.9	0.5	0.987
	10	76.7	2.8	0.998	82.7	1.3	0.989	92.7	3.0	1.000	11.8	0.3	0.996
	20	47.3	3.6	0.000	52.0	4.8	0.001	91.9	3.6	0.998	7.3	0.6	0.000
	30	28.7	4.3	0.000	32.0	5.6	0.000	90.7	4.0	0.979	4.5	0.7	0.000
Psyllium	0	90.7	1.6	–	88.7	1.3	–	102.3	1.5	–	13.5	0.2	–
	1	91.3	2.7	1.000	90.7	2.9	0.997	100.8	1.9	1.000	13.7	0.4	1.000
	3	90.0	1.8	1.000	88.7	2.5	1.000	101.6	1.5	1.000	13.4	0.3	1.000
	5	96.0	1.3	0.632	94.7	1.7	0.613	101.5	0.9	1.000	14.3	0.2	0.589
	10	64.7	4.4	0.000	66.7	4.5	0.000	97.0	1.3	0.823	9.8	0.7	0.000
	20	11.3	0.8	0.000	17.3	1.9	0.000	67.9	6.6	0.000	2.0	0.1	0.000
	30	0.0	0.0	–	–	–	–	–	–	–	–	–	–
Chia	0	62.0	3.1	–	64.7	3.4	–	96.0	1.0	–	9.4	0.5	–
	1	54.0	3.2	0.698	63.3	2.8	1.000	85.3	3.8	0.490	8.6	0.4	0.886
	3	58.0	5.4	0.984	68.0	5.5	0.992	85.0	1.9	0.453	9.2	0.8	1.000
	5	50.0	5.8	0.251	54.7	3.6	0.391	90.6	6.2	0.959	7.7	0.7	0.251
	10	51.3	1.3	0.379	66.7	2.8	0.999	77.7	4.6	0.040	8.5	0.2	0.825
	20	13.3	2.4	0.000	16.7	3.2	0.000	81.0	5.5	0.141	2.2	0.4	0.000
	30	0.0	0.0	–	–	–	–	–	–	–	–	–	–

Plants	Treatment time (s)	Length of root on the 3rd day (cm)			Length of root on the 6th day (cm)			Length of shoot (cm)			Length of seedling (cm)		
		Mean	SE	PHC	Mean	SE	PHC	Mean	SE	PHC	Mean	SE	PHC
Amaranth	0	1.4	0.1	–	2.0	0.1	–	1.2	0.1	–	3.2	0.1	–
	1	1.1	0.1	0.486	1.8	0.2	0.937	1.0	0.1	0.153	2.7	0.3	0.680
	3	1.6	0.2	0.835	2.3	0.3	0.979	1.3	0.1	0.866	3.6	0.4	0.942
	5	0.9	0.1	0.046	1.4	0.3	0.197	0.8	0.1	0.008	2.3	0.3	0.054
	10	0.4	0.0	0.000	0.6	0.1	0.000	0.3	0.1	0.000	0.9	0.2	0.000
	20	0.0	0.0	0.000	0.1	0.0	0.000	0.0	0.0	0.000	0.1	0.1	0.000
	30	–	–	–	–	–	–	–	–	–	–	–	–
Garden cress	0	3.5	0.2	–	7.1	0.2	–	3.4	0.1	–	10.4	0.3	–
	1	3.8	0.1	0.433	6.7	0.2	0.835	3.1	0.1	0.909	9.9	0.4	0.859
	3	3.3	0.2	0.898	6.7	0.3	0.856	2.9	0.1	0.999	9.6	0.4	0.942
	5	2.7	0.2	0.005	6.2	0.1	0.992	2.8	0.1	1.000	9.0	0.2	0.997
	10	1.2	0.2	0.000	2.0	0.2	0.001	1.2	0.1	0.003	3.2	0.3	0.001
	20	–	–	–	–	–	–	–	–	–	–	–	–
	30	–	–	–	–	–	–	–	–	–	–	–	–
Common flax	0	1.4	0.1	–	2.4	0.3	–	2.5	0.2	–	4.9	0.2	–
	1	1.4	0.1	1.000	2.1	0.2	0.933	2.8	0.3	0.973	4.9	0.4	1.000
	3	1.1	0.1	0.711	2.1	0.2	0.974	2.0	0.3	0.732	4.1	0.5	0.784
	5	1.3	0.2	0.986	2.0	0.2	0.784	2.6	0.2	1.000	4.6	0.3	0.998
	10	1.6	0.2	0.981	2.2	0.3	0.999	2.5	0.2	1.000	4.8	0.5	1.000
	20	0.6	0.1	0.002	1.0	0.1	0.001	1.1	0.2	0.004	2.1	0.3	0.000
	30	0.4	0.1	0.000	0.6	0.1	0.000	0.7	0.1	0.000	1.2	0.2	0.000
Psyllium	0	1.1	0.1	–	1.8	0.1	–	1.6	0.1	–	3.5	0.1	–
	1	1.1	0.1	1.000	1.9	0.2	1.000	1.5	0.1	0.395	3.3	0.3	0.999
	3	1.2	0.1	0.984	1.9	0.2	1.000	1.2	0.0	0.000	3.1	0.2	0.693
	5	0.9	0.0	0.425	1.7	0.2	0.979	1.0	0.1	0.000	2.7	0.3	0.079
	10	0.6	0.1	0.000	1.1	0.1	0.017	0.8	0.1	0.000	1.9	0.1	0.000
	20	0.1	0.0	0.000	0.2	0.0	0.000	0.1	0.0	0.000	0.3	0.1	0.000
	30	–	–	–	–	–	–	–	–	–	–	–	–
Chia	0	1.5	0.1	–	2.9	0.2	–	1.2	0.1	–	4.0	0.3	–
	1	1.3	0.1	0.877	2.5	0.2	0.909	1.1	0.1	0.999	3.6	0.3	0.948
	3	1.2	0.1	0.430	2.4	0.4	0.795	1.0	0.1	0.767	3.4	0.4	0.742
	5	1.3	0.2	0.587	2.2	0.3	0.353	1.3	0.2	0.878	3.5	0.4	0.837
	10	1.3	0.0	0.779	2.1	0.1	0.251	0.9	0.0	0.247	3.0	0.2	0.195
	20	0.2	0.0	0.000	0.4	0.1	0.000	0.2	0.0	0.000	0.6	0.1	0.000
	30	–	–	–	–	–	–	–	–	–	–	–	–

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Plants	Treat- ment time (s)	Weight of fresh root (mg)	Weight of fresh root (mg)	Weight of fresh shoot (mg)	Weight of fresh seedling (mg)	Weight of dried root (mg)	Weight of dried shoot (mg)	Weight of dried seed- ling (mg)
Psyllium	0	0.05	0.01	–	0.25	0.02	–	–
	1	0.07	0.02	1.000	0.28	0.01	0.710	0.030
	3	0.17	0.09	0.267	0.27	0.01	0.955	0.020
	5	0.11	0.03	0.943	0.30	0.01	0.199	0.020
	10	0.03	0.00	1.000	0.16	0.01	0.001	0.020
	20	0.00	0.00	0.958	0.02	0.02	0.000	0.020
	30	–	–	–	–	–	–	0.036
Chia	0	0.05	0.01	–	0.15	0.01	–	–
	1	0.04	0.01	0.981	0.15	0.01	1.000	0.030
	3	0.03	0.01	0.962	0.15	0.01	1.000	0.010
	5	0.04	0.01	0.999	0.12	0.01	0.657	0.010
	10	0.03	0.01	0.783	0.14	0.01	0.979	0.010
	20	0.01	0.01	0.096	0.04	0.04	0.000	0.010
	30	–	–	–	–	–	–	0.000

Plants	Treat- ment time (s)	R/S_length			R/S_fresh_weight			R/S_dry_weight			Seedling vigor index I. (cm)			Seedling vigor index II. (mg)			Seedling vigor index III. (mg)		
		Mean	SE	PHC	Mean	SE	PHC	Mean	SE	PHC	Mean	SE	PHC	Mean	SE	PHC	Mean	SE	PHC
Ama- ranth	0	1.7	0.1	–	0.64	0.05	–	0.890	0.270	–	2.1	0.2	–	0.08	0.01	–	0.000	0.000	–
	1	1.9	0.2	0.999	0.41	0.03	0.116	1.020	0.190	1.000	1.8	0.3	0.949	0.07	0.01	0.755	0.010	0.000	0.434
	3	1.7	0.1	1.000	0.57	0.09	0.976	0.670	0.050	0.999	2.8	0.4	0.363	0.09	0.01	1.000	0.010	0.000	0.350
	5	1.7	0.3	1.000	0.48	0.04	0.443	1.970	0.670	0.218	1.3	0.2	0.132	0.06	0.01	0.073	0.000	0.000	1.000
	10	1.8	0.2	1.000	0.44	0.11	0.241	0.980	0.340	1.000	0.3	0.1	0.000	0.01	0.00	0.000	0.000	0.000	0.168
	20	0.0	0.0	0.000	0.00	0.00	0.000	0.000	0.000	0.421	0.0	0.0	0.000	0.00	0.00	0.000	0.000	0.000	0.004
	30	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Garden cress	0	2.1	0.0	–	0.47	0.01	–	0.290	0.010	–	10.3	0.4	–	0.66	0.06	–	0.040	0.000	–
	1	2.2	0.0	0.999	0.16	0.01	0.954	0.230	0.020	0.619	9.6	0.4	0.893	0.69	0.03	0.583	0.040	0.000	0.886
	3	2.3	0.1	0.127	0.14	0.02	0.999	0.210	0.030	0.359	9.3	0.4	0.971	0.67	0.04	0.693	0.040	0.000	0.929
	5	2.2	0.0	0.849	0.17	0.02	0.652	0.230	0.060	0.755	8.9	0.2	0.998	0.71	0.01	0.441	0.030	0.000	1.000
	10	1.7	0.1	0.000	0.12	0.01	0.717	0.090	0.010	0.000	2.2	0.3	0.000	0.27	0.04	0.023	0.020	0.000	0.076
	20	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	30	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Common flax	0	1.0	0.2	–	0.20	0.02	–	0.280	0.010	–	3.8	0.2	–	0.77	0.03	–	0.070	0.010	–
	1	0.8	0.1	0.827	0.19	0.03	1.000	0.250	0.020	1.000	3.6	0.4	0.997	0.47	0.05	0.537	0.070	0.010	1.000
	3	1.1	0.1	0.998	0.43	0.09	0.009	0.460	0.070	0.994	3.3	0.5	0.946	0.46	0.04	0.502	0.060	0.010	0.999
	5	0.8	0.1	0.831	0.17	0.02	0.995	0.200	0.010	1.000	3.9	0.4	1.000	0.68	0.05	0.998	0.090	0.010	0.878
	10	0.9	0.1	0.989	0.19	0.01	1.000	0.200	0.010	1.000	3.9	0.5	1.000	0.72	0.04	1.000	0.090	0.010	0.849
	20	1.0	0.2	1.000	0.00	0.00	0.154	0.000	0.000	0.548	1.1	0.2	0.000	0.28	0.28	0.085	0.030	0.030	0.299
	30	0.9	0.1	0.989	0.00	0.00	0.091	0.000	0.000	0.240	0.4	0.2	0.000	0.08	0.08	0.005	0.010	0.010	0.390

Plants	Treat- ment time (s)	R/S_length	R/S_fresh_weight	R/S_dry_weight	Seedling vigor index I. (cm)	Seedling vigor index II. (mg)	Seedling vigor index III. (mg)										
Psyllium	0	1.1	0.1	0.23	0.06	0.340	0.120	—	3.1	0.1	—	0.02	—	0.030	0.010		
	1	1.3	0.1	0.976	0.24	0.06	1.000	0.430	0.080	0.983	3.0	0.3	1.000	0.31	0.03	0.986	0.884
	3	1.6	0.1	0.289	0.60	0.28	0.274	0.460	0.030	0.926	2.7	0.2	0.857	0.39	0.10	0.390	0.816
	5	1.6	0.1	0.134	0.34	0.08	0.990	0.380	0.090	1.000	2.6	0.3	0.573	0.38	0.05	0.500	0.883
	10	1.5	0.1	0.467	0.21	0.02	1.000	0.460	0.040	0.915	1.3	0.2	0.000	0.13	0.01	0.267	0.180
	20	1.6	0.3	0.225	0.00	0.00	0.892	0.000	0.000	0.324	0.1	0.0	0.000	0.00	0.00	0.003	0.009
	30	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Chia	0	2.4	0.1	—	0.29	0.03	—	0.160	0.050	—	2.6	0.3	—	0.13	0.01	—	—
	1	2.2	0.1	0.983	0.24	0.03	0.986	0.250	0.040	0.812	2.3	0.3	0.970	0.12	0.01	0.996	0.512
	3	2.4	0.2	1.000	0.21	0.06	0.886	0.190	0.020	0.999	2.4	0.4	0.995	0.13	0.03	1.000	0.551
	5	1.7	0.1	0.079	0.35	0.06	0.974	0.190	0.040	0.999	2.0	0.3	0.519	0.09	0.01	0.347	0.246
	10	2.4	0.1	1.000	0.21	0.04	0.877	0.190	0.030	0.999	2.0	0.1	0.631	0.11	0.01	0.959	0.493
	20	2.4	0.3	1.000	0.00	0.00	0.035	0.000	0.000	0.693	0.1	0.0	0.000	0.01	0.01	0.000	0.054
	30	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

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Data Availability No datasets were generated or analysed during the current study.

Declarations

Conflict of interest All authors declare no conflict of interest to disclose.

Ethical Approval Not applicable.

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