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Germination and Growth Improvement of Some Micro-Greens under the Influence of Reactive Species Produced in a Non-Thermal Plasma (NTP)

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Abstract: Micro-greens or sprouts are commonly used in the diet of many cultures owing to their health benefits. In this work we use a non-chemical solution method to stimulate the germination and growth of seeds used for sprouting, based on non-thermal plasma discharge (NTP). Such a technology could represent an alternative not only for reducing the production costs for growing micro-greens but also as a pre-sowing method for slow germinating species or those under draught stress. To evaluate the efficiency of the treatments, *Brassica oleracea* and *Lepidium sativum* seeds were exposed to a non-thermal plasma discharge produced in atmospheric air in different conditions. The strongest modifications were recorded when the discharge was produced in a closed environment when the reactive species produced in air remained in high concentrations near the seeds. The garden cress exhibited stronger modifications, with a decreasing of the water contact angle of the seeds by up to about 14%, which means an increase of the hydrophilicity of the surface of the seeds. The stimulation of the growth was evaluated as an increase of the average stem length of (9 $\pm$ 0.4)% and of the root length of (38 $\pm$ 0.5)% as compared to sprouts grown from untreated seeds. This indicated that the reactive species were not only interacting with the surface of the seeds as proved by electron microscopy imaging but also penetrated inside the seeds, activating biological pathways that lead to the stimulation of growth in this case. A noticeable influence produced by the reactive species was also reflected in the biochemical results, where the analysis of the chlorophyll pigments indicated strong modifications, especially under the intensified action of the reactive species. The results prove an important contribution from the reactive species and show the possibility of using this technology to improve the growth of these micro-greens, reducing production time and even presenting the possibility of treating packaged seeds.

Keywords: seed treatment; sprouting; micro-greens; reactive species; plasma agriculture

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1. Introduction

Plasma agriculture is a relatively new and fast developing field in which plasma, an ionized gas, serves for the processing of different biological materials with the purpose of changing plant properties and behavior [1–36]. In particular, atmospheric pressure plasmas are used for such applications, meaning that a high electric field is applied to air, producing a discharge. Since the air contains at least oxygen and nitrogen, an atmospheric plasma will contain many reactive species along with the ions, electrons, neutrals, and emitted radiation. Atmospheric plasmas have a complex chemistry driven by electrons, are versatile, are produced in relatively simple configurations that can be easily implemented in large-scale applications, and operate at low temperature, which is compulsory when treating biological materials.

The advantages of NTPs for processing biological materials have been shown in many studies, with the most important being that it is a green technology that is environmentally and user friendly and does not use dangerous chemicals such as fertilizers and pesticides [1–7]. The benefic effects related to plants have led to the fast development of a new research field—"plasma agriculture". In the current situation of climate change, with increasing population, it is necessary to find and perfect novel alternative technologies to sustain food production by increasing yield, quality, and shelf-life. NTP is a good alternative that not only has proven to stimulate the germination and growth of plants but has been shown to reduce the microbiological load from the surface of the seeds and plants [5,6,11,21]. NTP-based stimulation of germination and sprouting, including through the indirect action when water is treated and plasma activated water (PAW) is obtained, has been reported for different seed species, such as radish [2,36], carrot [9], lentil [11], soybean [27], pea [31], spinach [33], rice [13,28,34], maize [15,18,24], buckwheat [16,23], corn [20], and others [21–26,30,32,35,37–39]. With minor exceptions, the majority of reported results are positive, including stimulation of germination and plant development, improved harvest, and improvement of the benefic compound content. The negative results show inhibition of germination and growth, and also negative effects on the DNA in some conditions [12,13,17,25]. Despite being unappealing for publishing, the inhibitory effects must be reported and considered, so that lack of knowledge regarding the mechanisms of interaction between plasma and different species of plants can be addressed and fine-tuned so as to obtain the desirable effects. The plasma species (e.g., ions, excited molecules, other reactive species), radiation, and electromagnetic field are physical or/and chemical stressors for the plants, just as salinity, radiation, or draught, for example. These stress factors induce positive and negative effects; it is reasonable to expect and understand both, in order to identify the optimum conditions for the expected results in each case. Moreover, plasma has proven to act as a restoring germination factor in the case of rice seeds affected by heat stress [28], and should be considered as future technology in sustainable agricultural production.

In this work, we focus on studying the behavior of *Brassica oleraceae* (broccoli) and *Lepidium sativum* (garden cress) under atmospheric pressure NTP treatment in extended conditions. These species are commonly used to produce sprouts for consumption, a dietary habit very common in Asia and America, with only recently increasing interest in Europe. The appealing aspect of micro-greens consumption is related to their content of antioxidant substances, health promoting compounds, minerals (potassium, calcium, phosphorus, iron, manganese, zinc), and nutrients, thus preventing anemia and addressing micronutrient deficiencies [40–42]. It is a benefic species, with its extracts having also many medicinal uses (e.g., antirheumatic, diuretic, management of asthma, anti-inflammatory, blood coagulant) [43,44].

Despite the abundance of previous studies, there is still little known about the mechanisms involved in the process, for which we state the necessity of a deeper analysis of the interactions between plasma and seeds, and the necessity of reporting the inhibitory effects plasma has in some conditions on the treated seeds. The reported growth and physiological effects are actually responses to the synergistic physical action of plasma components; thus, it is normal to expect different responses between species, as we previously discovered in the case of Japanese radish and broccoli sprouts [39]. Therefore, the purpose of this work is to deepen the analysis of the interaction mechanisms between atmospheric plasmas and different sprouting seeds, to bring some insights into the process by using new exposure conditions with enhanced effects of the oxygen and nitrogen reactive species (RNOS). Due to the complexity of the synergistic factors that can modify the seeds and produce an effect on their germination and growth, it is difficult to separate out each contribution. However, we aim to study the behavior while keeping the produced reactive species in a closed environment, where the treated seeds are compared with the case when these reactive species diffuse freely in the environment.

2. Materials and Methods

2.1. Experimental Device

In this work, we used the same configuration as reported previously [39] with a flexible electrode as schematically represented in Figure 1. It produces a surface dielectric barrier discharge that is a non-thermal plasma (NTP); a similar configuration proved to be efficient for microorganism inactivation on the surface of wrapped medical equipment [45], in liquid bacteria [46], and in meat [47]. A metallic mesh (5 × 5 cm) representing the power electrode was placed on top of a glass plate used as dielectric. On the other side of the glass, there was a metallic tape ground electrode.

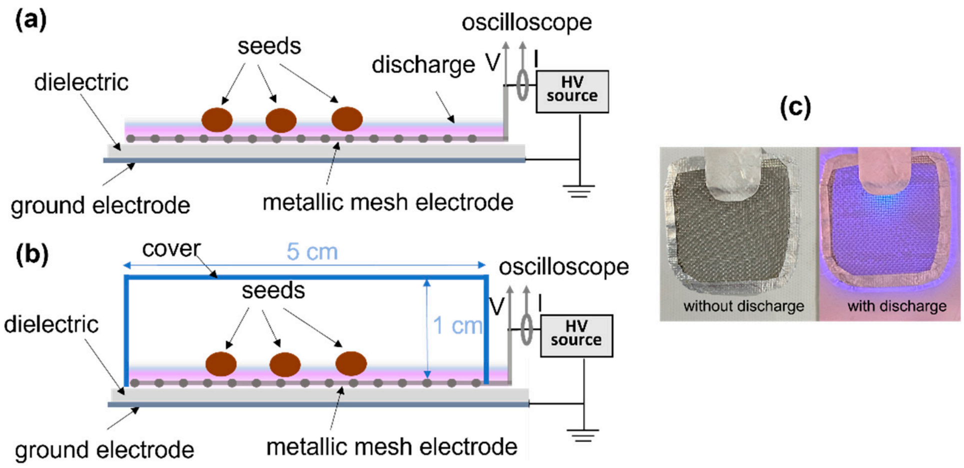

Figure 1. Schematic representation of the flexible configuration used for the seed treatments: (**a**) direct plasma, (**b**) with cover, (**c**) picture of the electrode from above without and with discharge.

To ignite the discharge, a high-voltage (HV) signal source was used, HV18K603AC, which produced a 10 kHz signal of up to 18 kV peak-to-peak value. The discharge was produced on top of the mesh electrode in open air, without the use of any other gas. For the experiments presented here, signals up to 14 kV were used. The I-V characteristics were monitored in all studied cases using an HV probe (Tektronix, P6015A, Beaverton, OR, USA) and a current probe (Pearson, 4100, Palo Alto, CA, USA) connected to a RIGOL DS2072A oscilloscope. The emission spectra of the discharges were monitored using Ocean Optics HR2000.

2.2. Biological Material and Treatment

The seeds were commercially procured. Plasma treatment represents the simultaneous action of several factors among which reactive species might play a very important role, especially in the case of atmospheric air discharges. Thus, we chose to perform the seed treatments in two configurations: in one, the seeds were simply put on the mesh electrode—direct exposure; in the second, a cover was used to keep the reactive species produced in plasma from diffusing in air far from the sample, thus enhancing the effects produced by the reactive species—with cover. Seeds were placed in one layer over the mesh electrode (approx. 100 seeds) without any prior preparation. All experiments were performed in triplicate.

2.3. Seeds Surface Morphology and Wettability

The surface of the seeds was analyzed by environmental scanning electron microscopy (ESEM) using a FEI Quanta 450 (Thermo Fisher Scientific, Hillsboro, OR, USA) system in low vacuum mode (100 Pa), so that the seeds would not to shrink due to dehydration in high vacuum conditions. The surface of the seeds mounted on aluminum stubs with adhesive carbon double tape was imaged with a 15 kV electron accelerated beam without any prior preparation, because ESEM allows the imaging of non-conductive samples.

The wettability of seeds' surface was determined immediately after the treatments using the water drop method. A total of 1µL of pure water was dropped on the surface of the seed; then, a picture was taken and analyzed to determine the water contact angle using imageJ software with the drop snake plugin [48]. For the cress seeds, ESEM images were analyzed with the same software using the roughness estimation plugin, because there was no obvious change of the surface morphology of exposed seeds when analyzed by electron microscopy. The presented results were statistically processed based on the measurements from each batch of seeds in each condition.

2.4. Biometric Measurements

After performing the treatments in the above-mentioned conditions, the seeds were put in containers with 2 mL of water in each. The average temperature of the environment during the experiment was 20 °C, while the relative humidity varied between 45 and 60%. The germination potential for each treatment condition was evaluated starting on day 3 after the initial treatment; it represents the number of germinated seeds divided by the initial number of seeds times 100. The biometric measurements (stem length and root length) were done on the 7th day after plasma treatment. In all cases, values were compared to the control, untreated samples. A statistical analysis was made with data being compared by unidirectional analysis variance (ANOVA).

2.5. Biochemical Measurements

After 7 days, the chlorophyll contents (chlorophyll *a*, *b*, and total carotenoids) in untreated and NTP-treated samples in different conditions were evaluated using a trichromatic spectrophotometric protocol [49]. The pigments were extracted by grinding 5 g of fresh vegetal product, then filtrating the sample using 96% ethanol. The resulting solution was analyzed by measuring the absorption from 200 nm to 900 nm using a spectrophotometer (Specord 210 Plus, Analytikjena, Jena, Germany). The concentration was calculated using the absorbances of the sample extracts at 665, 649, and 470 nm against a blank sample. In each case, three repetitions were carried out; average values, standard deviations, and *t*-test were determined.

3. Results and Discussion

The electrical characteristics of the discharge were determined (Figure 2a), and the discharge power was estimated from current—voltage characteristics measured for different HV applied signals; the dependence between the value of the power and the peak-to-peak value of the applied HV was found to be almost linear. The data are presented in Figure 2b. The obtained values for the power are consistent with others reported for similar discharges, as are the emission spectra [45–47,50]. The temperature of the flexible electrode during the treatments did not exceed 34 °C, as measured with an infrared thermometer; thus, any thermal damage of the seeds during the treatments can be excluded.

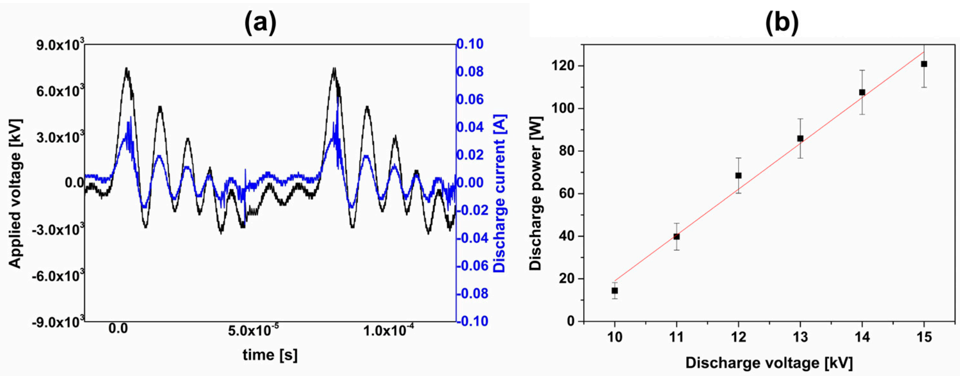

Figure 2. (**a**) Typical IV characteristic of the discharge and (**b**) estimation of the NTP power as a function of the peak-to-peak discharge voltage value from electrical measurements.

Optical emission measurements (Figure 3) aided us in identifying some of the reactive species produced in the discharge, species that impacted the seed. The radiative species identified are -OH radical, N_2, and N_2^+. Oxygen reactive species were produced in the discharge (ozone generation was felt during the treatments), but oxygen emitting species tend to lose energy before being detected, thus explaining their absence in the emission spectrum.

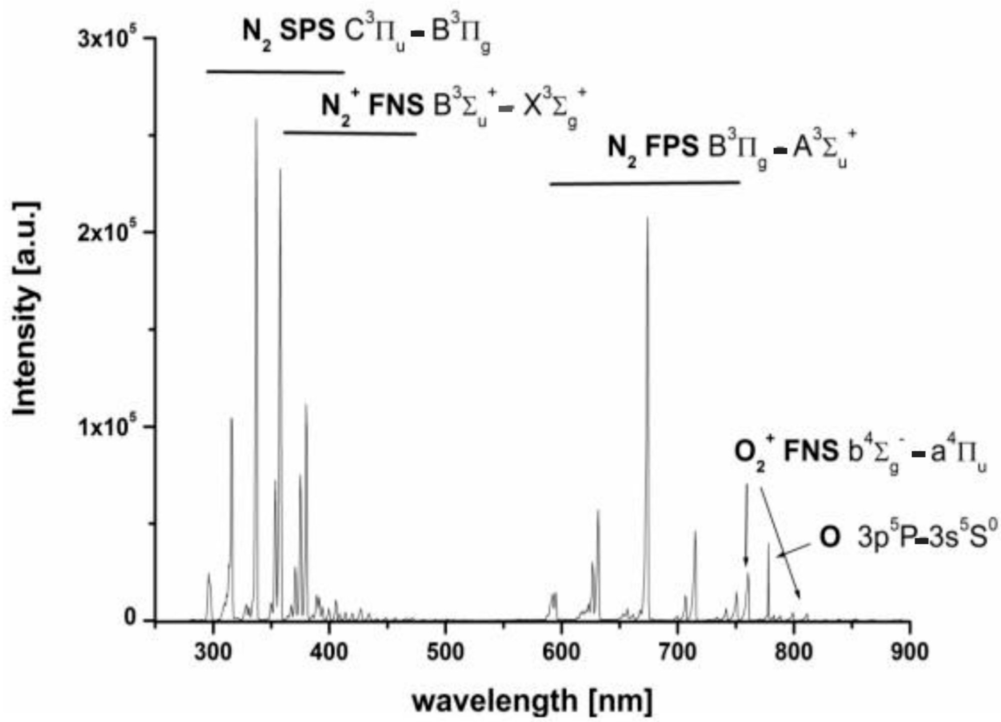

Figure 3. Emission spectrum indicating some of the radiative reactive species produced.

With cover, the germination potential of broccoli seeds is smaller in all voltage cases, as can be seen from Figure 4a, similar to our previous work [39]. For processing periods of 60s and longer, the germination process is inhibited, especially for voltages higher than 10 kV, which corresponds to an approximate power density of about 0.03 W/cm^3. Still, small but significant increase of the stem length of the sprouts was noticed for the 9 kV, 60s treatment and 10 kV, 30s conditions. For short exposure intervals, there is no linear correspondence between stem length or root length and voltage or discharge power (Figure 4b,c). With cover, the inhibition effects are much stronger than for direct treatment without cover, indicating a strong contribution of the reactive species.

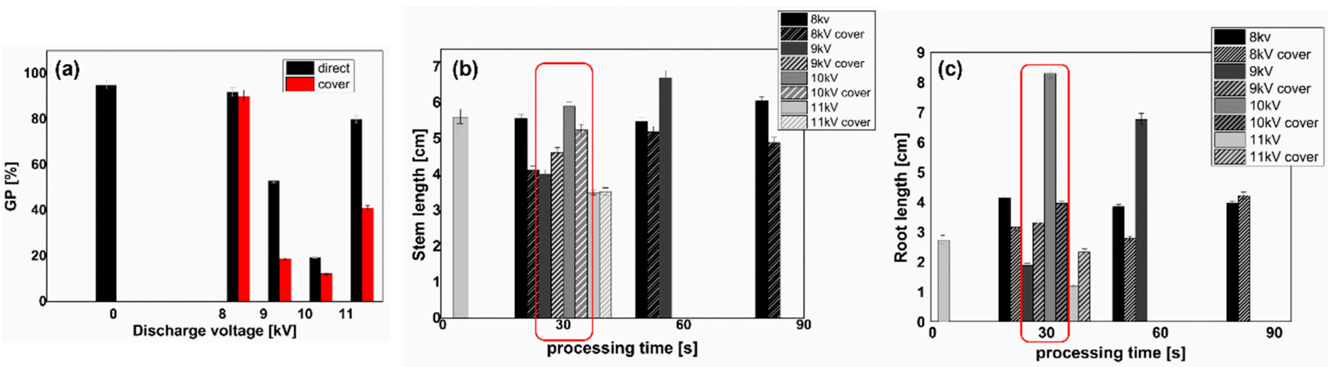

Figure 4. (**a**) Germination potential for broccoli seeds for 30s treatments with and without cover: (**b**) stem length and (**c**) root length for broccoli seeds ($p < 0.005$).

To explain this phenomenon, we analyzed the surface of the seeds using ESEM. The images in Figure 5 can be correlated with the above-mentioned results; Figure 5f indicates drastic changes in the surface morphology under 14 kV with cover treatment of the seeds. In these treatment conditions, we also saw a decrease of the water contact angle. Overall, using high discharge voltages and the concentrated action of reactive species, the surface of the broccoli seeds is first cleaned; then, the etching process reaches the inside layers, decreasing the water contact angle, facilitating the diffusion of reactive species towards the inside of the seed, and stimulating the growth of sprouts, or for intense treatments (longer and high voltage NTP), rupturing of the cell walls on the epidermis with exposure of inner layers is evident (Figure 5f). The changes are more obvious for broccoli than for cress. Similar disintegration of the outer layer of the seeds and irregular shape agglomerations (as seen in Figure 5) have been also reported in DBD-treated quinoa seeds [51], low pressure treatment processing of Arabidopsis thaliana [52], and wheat treatment [53]. With cover, the broccoli seed surface outer cell layer is removed (Figure 5e), while for cress, although a change is not obvious (Figure 5g–i), the evaluation of roughness, as performed using imageJ, indicates only slight modification for simple sDBD treatment (83.01 ± 1.30) compared to untreated samples (88.77 ± 1.30), and stronger modification for the concentrated reactive species treatment (Figure 5i) (113.70 ± 1.30).

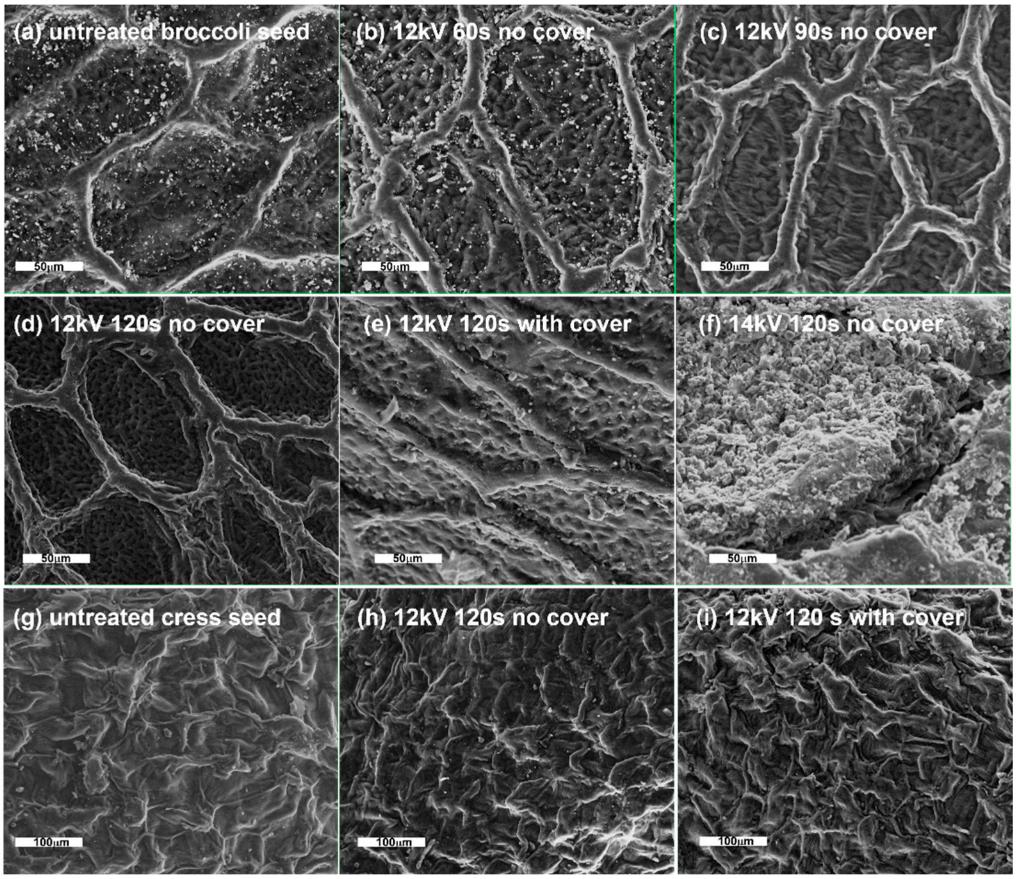

Figure 5. ESEM images of the surface of untreated and treated broccoli (**a**–**f**) and cress (**g**–**i**). (**a**) untreated broccoli seed, (**b**) broccoli seed treated with 12 kV NTP for 60s without cover, (**c**) broccoli seed treated with 12 kV NTP with cover, (**d**) broccoli seed treated with 12 kV NTP for 120s without cover, (**e**) broccoli seed treated with 12 kV NTP for 120s with cover, (**f**) broccoli seed treated with 14 kV NTP for 120s without cover, (**g**) cress seed untreated, (**h**) cress seed treated with 12 kV NTP for 120s without cover, and (**i**) cress seed treated with 12 kV NTP for 120s with cover.

For the treatment conditions for which we found the strongest modifications of the seed surfaces, we also evidenced a decrease of the water contact angle, as can be seen in the results in Table 1. In some conditions, we see an increase of water contact angle, especially for the intensified reactive species conditions, with up to 14%, while for other conditions (high voltage, long treatment time, and concentrated reactive species), there is a decrease with about the same percentage. The increase in hydrophilicity of the seed surface is similar to other reports and accompanies an increase of wettability and water uptake capacity, both through physical (etching) and chemical interactions. The values smaller than the control recorded in this experiment might be caused by strong damage of the epidermis, as confirmed by the ESEM imaging.

Table 1. Water contact angle of cress seeds treated in different conditions: without cover and with cover.

Voltage	DP/IP	0	30s	60s	90s	120s
11 kV	DP/	101.3	106.3	105.2	108.4	106.4
	/IP		107.5	110.2	99.5	100.2
12 kV	DP/		106.0	107.5	101.1	107.5
	/IP		109.1	116.0	100.9	101.8
13 kV	DP/		102.4	101.2	100.5	100.4
	/IP		113.4	115.6	109.1	100.5
14 kV	DP/		93.8	91.5	90.3	87.1
	/IP		88.9	97.3	87.0	83.2

Overall, using high discharge voltages and the concentrated action of reactive species, the surface of the broccoli seeds is bombarded by the reactive species, which produce physical and chemical changes of the outer epidermis, decreasing the water contact angle. Similar changes have been reported in other plasma processing experiments of seeds [26]. The effects seem to strongly depend on the type of treatment and also on the species treated, since different species would have different morphology of the seed coats. In our case, a section through the seed was performed to assess the dimension of different layers; it shows that the broccoli seed has a thicker coat seed towards the hilum and thinner towards the apex, while garden cress has a thick coat all over the seed, much thicker than broccoli (Figure 6).

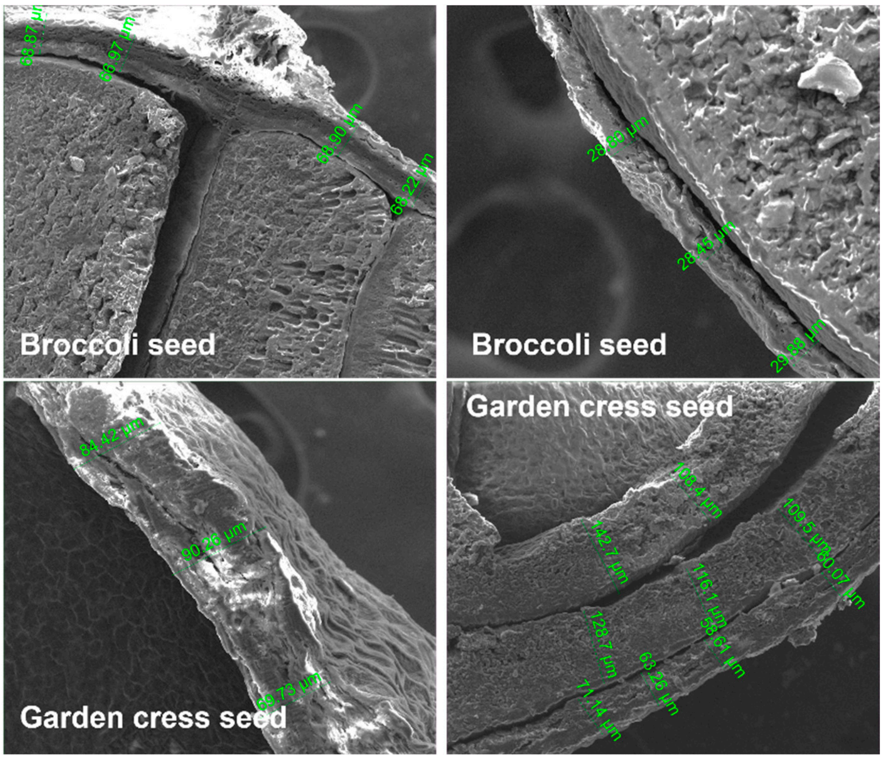

Figure 6. ESEM images of seed section for broccoli and garden cress seeds.

The biometric data for cress seeds presented in Figure 7 shows that the length of the stems and roots increases at 11 kV and starts decreasing at 12 to 14 kV, with a non-linear behavior; using the cover, we obtain an improvement of plant length for almost all conditions, higher than for the no cover case, indicating the importance of reactive species contribution. The same data indicate an overall increase of the stem length with processing time; however, it starts with an inhibition of growth stimulation. Data shows that most of the treated seeds up to 120s will grow in sprouts of smaller length than untreated seeds. This behavior might be an abiotic stress response. The results seem to be less dependent on the dissipated energy and more on the intensified action of reactive species. The electromagnetic field could also have a small contribution to the stimulation of growth, as other studies have found, but without influencing the germination [16,54,55]. Still, this might produce modifications at the cell level by increasing the production of reactive oxygen species in the seeds. In our case, the exposure with and without cover was performed in the same electromagnetic field conditions; thus, comparing between the same voltage conditions with and without cover, the effects can be directly attributed to the reactive species confined near the seeds in the case of using the cover.

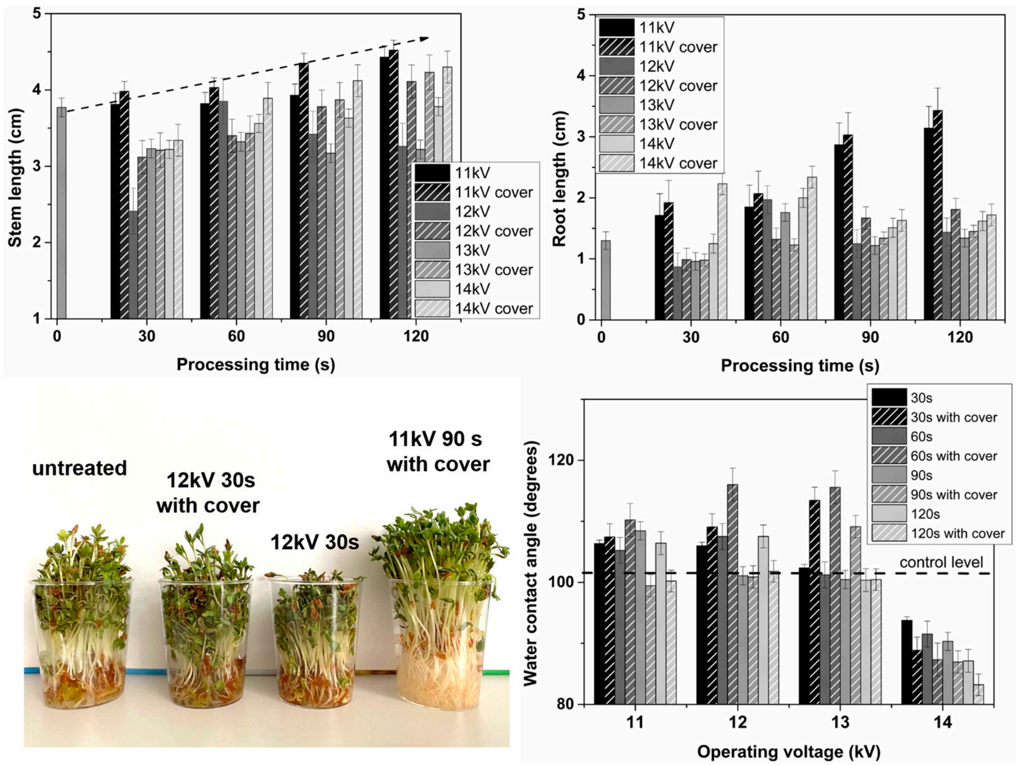

Figure 7. Biometric measurements for cress sprouts (stem and root length) and water contact angle for the seeds after treatment in different conditions.

The reactive species are known to play key roles in different stages of the seed life, with both detrimental and positive effects. Recent research has indicated that ROS can be beneficial, influencing the germination through different signaling pathways and regulating the growth [56–59]. Not only oxygen reactive species but also nitrogen reactive species seem to have an important role; researchers found nitrogen reactive species to better stimulate the germination of wheat in the case of higher RNS concentrations, but longer shoots were found for higher ROS [59]. Based on the emission spectrum and other literature reports, we expect high concentrations of reactive species such as O_3, $^1O_2$, $^{\cdot}O_2$, $^{\cdot}NO$, $^{\cdot}OH$, and $N_2^{\cdot}$ [60–63]. These species introduce oxidative and nitrosative stress into the exposed cells and lead to the production of other reactive species inside the cells as a response to the initial stress. This process is very common as a response to other stressors in plants, such as drought or salinity, influencing different stages of plant development and physiological characteristics [64]. During the treatments with cover, long life RONS would be concentrated near the samples. The same non-linear trend is also common as a stress-induced change: for a low stress impact on the seeds, favorable mechanisms of growth and enhancement of physiological parameters are in place to counteract the stressor, but in the case of high stress, the physiological processes are disturbed, which results in the inhibition of germination, growth, synthesis of bioactive compounds, etc. [64].

Despite the thick and little-affected outer epidermis of cress, reactive species seem to reach the inside of the seed, stimulating the growth of the sprouts. The physical penetration depth is less important than the extension of the chemically induced effects, since RONS are strongly reactive and trigger the production of other reactive species inside the cells. The stimulation effects found in this work, higher in the case of concentrated reactive species exposure, could be explained through this kind of mechanism, underlining the importance of the reactive species in plasma more than the contribution of other components. From the latter, the electrical field could also have an influence; it has been long known that the exposure to electric fields alone might influence the germination in some cases [16,54,55], though in this situation, probably to a smaller extent.

The chlorophyll content of radish sprouts shows minor variations, including the case of concentrated reactive species exposure, while the most affected parameter is the germination potential and thus the viability of the broccoli seeds. Cress sprouts exhibit a different behavior: while the viability of the seeds remains close to 100% and the surface morphology of the seeds is less affected, we measured stronger variations of the biochemical parameters than in the case of broccoli, and stronger for the case with cover compared to no-cover treatments. The latter cases differ in behavior as well: for no cover, both Chlorophyll *a* and *b* concentrations slightly increase with treatment time, while when the seeds are exposed to concentrated reactive species, the concentrations of both pigments strongly decrease, as can be seen in Figure 8.

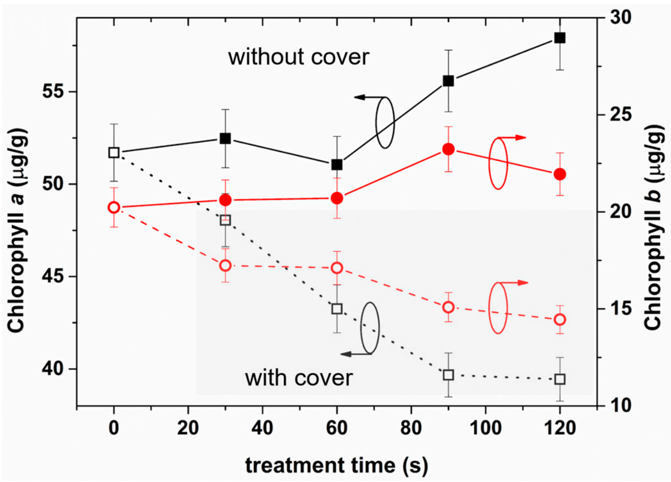

Figure 8. Concentrations of Chlorophyll *a* and Chlorophyll *b* in cress sprouts for seeds treated without cover (full squares—Ch *a*, full circles—Ch *b*) and with cover (empty squares—Ch *a*, empty circles—Ch *b*).

The chlorophyll pigment concentration changes in Figure 8 confirm the important contribution of the reactive species. These physiological responses come from the chemical and less physical interaction of plasma-produced reactive species at the cell membrane level and probably inside the cell, activating signaling factors, changing the membrane transport, and producing oxidative stress. Much higher concentrations of chlorophyll pigments were also found in the case of maize seed treatment in the presence of nitrogen reactive species, with these seemingly having a crucial role in this effect [18,59].

4. Conclusions

The processing of seeds in NTP with and without the concentrated action of plasma-produced reactive species indicates the importance of the reactive species in the reported effects on the germination and growth of micro-greens. The behavior of different species is different probably due to the difference in their morphology and biology.

The effects evidenced are actually responses to abiotic stress that clearly influence the growth (changes of germination potential, biometric values), physiology (changes in the concentration of the photosynthetic molecule concentrations of developing sprouts), and molecular biology of the samples. Difference in the response to plasma and reactive species treatment could be explained through the difference in the tolerance mechanisms within species. The results show good premises for using NTP as a convenient and sustainable technology in stimulating the growth of micro-greens in a reduced timeframe. More studies need to be performed to better quantify the effects of reactive species produced in NTP, and also to deepen the knowledge on other biochemical parameters that could be influenced by these treatments, such as the content of antioxidant compounds or important enzymes.

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