PAPER

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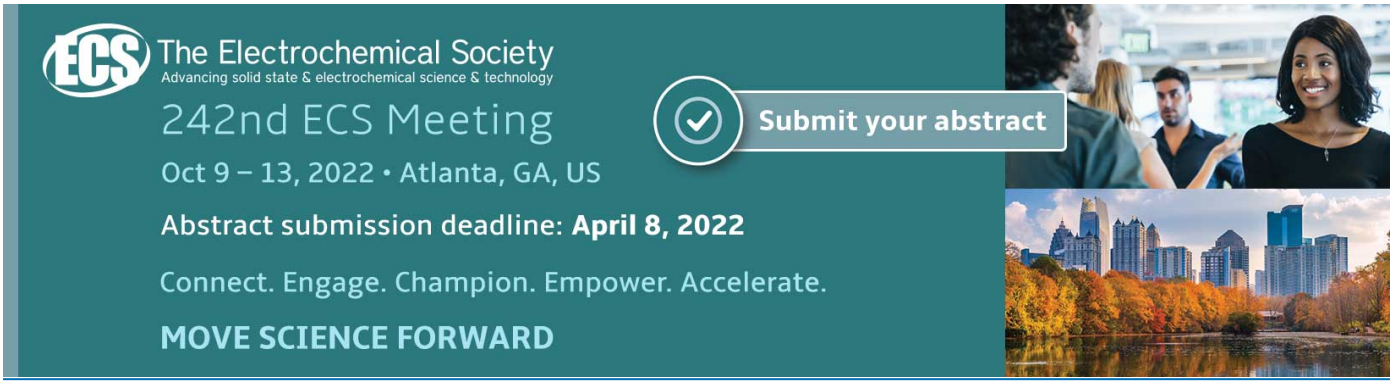

IOP Publishing Journal of Physics D: Applied Physics

J. Phys. D: Appl. Phys. **54** (2021) 325205 (13pp) https://doi.org/10.1088/1361-6463/abfefc

Factors triggering germination in plasma-activated cotton seeds: water imbibition vs. reactive species' formation

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Received 17 February 2021, revised 16 April 2021
Accepted for publication 7 May 2021
Published 8 June 2021

Abstract

Plasma technology has emerged as an efficient, simple, and eco-friendly method for activating seed germination processes. Most studies on this subject have focused on the morphological and wetting effects on the surface state of seeds and attributed the improvement in germination occasionally found to the induced hydrophilicity and a higher water imbibition rate. Recently, the involvement of reactive oxygen and nitrogen species in the germination process has also been highlighted. In this work, we study the effect of a very low-power cold atmospheric air-plasma treatment on the germination and sterilization of cotton seeds in normal (i.e., water abundant) and simulated drought conditions. Optical emission spectroscopy of the plasma has revealed the formation of different excited species of molecular nitrogen and oxygen, as well as OH and NO radicals that are deemed responsible for the chemical functionalization and slight morphological changes observed on the surfaces of cotton seeds. The physicochemical changes of the seed surface were analyzed by scanning electron microscopy, energy-dispersive x-ray spectroscopy, Fourier-transform infrared spectroscopy (FT-IR), and x-ray photoemission spectroscopy. This latter technique has shown the formation of a high surface concentration of oxygenated (i.e. $–CO_x$, –C–OH, $–O_x$) and oxinitrided (i.e. $–NO_x$) functional groups which disappear from the surface upon exposure to vapor or liquid water. This process must entail the diffusion of these species (together with that of potassium ions that were plasma segregated to the surface) into the interior of seeds. The fact that the water uptake capacity of seeds is not significantly modified by the plasma treatment suggests that the observed in-diffusion of active oxygen and nitrogen species is an important factor in the enhancement of germination capacity induced by plasma activation, particularly under conditions of water scarcity, where germination differences of more than 60% could be found between pristine and plasma-treated seeds. The

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analysis by FT-IR of the adsorption of deuterated water (D_2O) molecules is proposed as a useful procedure to directly monitor water uptake/release processes from seed surfaces.

Supplementary material for this article is available online

Keywords: plasma treatment, seed germination, seed sterilization, cotton, RONS diffusion

(Some figures may appear in colour only in the online journal)

1. Introduction

Plasma activation of seed surfaces is a hot topic with direct agricultural implications for the adaptation of production to global demands [1, 2], particularly under adverse drought and climate change conditions [3]. The food chain, from primary production to retail commercialization, the use of medicinal plants for the preparation of drugs and cosmetics and textile production (the latter led by the cotton industry) are severely affected by environmental conditions. To reduce the dependence on climate effects, a direct approach is to modify the genetics of plants (i.e., entailing modifications in germination capacity, the size of the plant, and other characteristics) to make them more resilient to drought, particularly during seed sowing periods [4]. A second approach, in which the use of plasmas may play a significant role, involves the application of surface activation processes to accelerate and enhance germination [5–9].

Seeds need specific conditions to germinate. Restrictions to seed germination may arise from either dormancy (seeds do not germinate, even if they have favorable environmental conditions) or quiescence (a delay in seed metabolism, a survival mechanism developed to adapt the seed response to certain adverse conditions). In the first case, dormancy can be interrupted by reactive oxygen or nitrogen species (ROS, RNS) or a combination of both (RONS) [10]. In the second case, water absorption and the triggering of specific internal physiological and biochemical processes are required to activate germination [4].

Most conventional activation methods of seed germination use chemical agents such as fungicides, organic and inorganic acids or pesticides that present significant risks to the environment and are harmful to human health [11]. Physical treatments such as ionizing radiation, radioisotopes or thermal processes are less polluting but, in general, they are also in general rather complex and expensive [12, 13]. Unlike these methods, plasma technology, particularly operated at atmospheric pressure, is an effective and relatively cheap physical method that has demonstrated the capacity to improve seed germination [14–16]. For example, it has been reported that plasma activation contributes to the acceleration/enhancement of germination, even in drought conditions, or to break dormancy [17] and induce an efficient sterilization (i.e., the removal of microorganisms, bacteria, fungi and biofilms) [18] while retaining plant characteristics and nutritional capacity [2]. The reported surface physicochemical changes that take place in parallel to this enhancement of germination induced by plasma include the modification by etching of the surface topography, changes in composition and hydrophilicity and the modification of the water absorption capacity [4, 5, 17–21]. Despite the ample literature on the subject, as presented in table 1, the key factors involved in the promotion of seed germination upon plasma treatment are not yet completely clear.

In this work, trying to shed some light on this complex issue, we study the existence of possible links between the atmospheric plasma activation of cotton seeds and their capacity to absorb water and/or to promote other effects that may trigger germination. The plasma activation of cotton seeds has previously been studied by Wang *et al.* [5] although these authors only explored the effect of plasma on seed surface characteristics, assuming that the increase in water uptake capacity was the main factor responsible for the germination improvement. In this work, we present a thorough characterization of the surface state of plasma-treated cotton seeds using a variety of physical techniques, including Fourier-transform infrared spectroscopy (FT-IR) analysis combined with the adsorption of D_2O (D: deuterium) and an x-ray photoemission spectroscopy (XPS) investigation of the chemical surface state of seeds before and after plasma treatment. We then try to correlate the results of this physicochemical analysis with changes in the germination capacity and the sterilization state. In this investigation, we find that plasma-treated cotton seeds are effectively sterilized and their germination rate is accelerated, even when treated at low applied powers, particularly under simulated drought conditions. The fact that water uptake capacity from the vapor or liquid phases is only slightly modified by the plasma treatment has prompted us to tentatively propose that alternative factors involving the surface functionalization and the inward migration of plasma-generated surface species, among which, some RONS, and/or the partial removal of germination-retarding factors (e.g., abscisic acid (ABA) [23, 24]) might contribute to the observed acceleration of the germination rate following after plasma treatments.

2. Method

2.1. Cotton seeds

Certified cleaned cotton seeds (Vitrosur S.A.) of the ELPIDA R1 variety were used for this study. This conventional species of cotton is appreciated because of its high productivity in humid soils, particularly if the first irrigation occurs soon after sowing. Typical cotton seeds have an approximate size of $2 \times 5 \times 10\ \text{mm}^3$, an internal structure made of successive layers and a rather thick bark, about 200 μm thick (estimated by scanning electron microscopy (SEM)) made of hemicellulose,

Table 1. State of the art with relevant examples in dielectric barrier discharge (DBD) plasma agriculture applications, particularly in cotton culture. The experimental conditions as well as the proposed main plasma factor(s) responsible for the stimulation of germination are included for comparison with those of this work.

Seed [ref]	Air pressure	Electrical parameters	Time	Main plasma effects
Tomato, pepper [22]	1 atm	0.2 kHz, 21 kV, 130 W cm^{-3}	10 min	Surface functionalization
Quinoa [20]	0.49 atm	1 kHz, 4.1 kV, 3.5 mA, 6.4 W	15 min	Surface functionalization/Species migration
Wheat [21]	1 atm	16 kHz, 20 kV, 30 W	2 min	Hydrophilicity/Surface oxidation
Cotton [16]	1 atm	1 kHz, 19 kV	27 min	Hydrophilicity
Cotton [5]	1 atm	1 kHz, 19 kV, 10 mA	27 min	Etching/Surface oxidation
Cotton [this work]	0.49 atm	1 kHz, 4.1 kV, 1.9 mA, 1 W	15 min	Surface functionalization/Species migration/ Removal of retarding elements

cellulose and lignin [25, 26]. A schematic of the seed's structure and an energy-dispersive x-ray spectroscopy (EDX) analysis of the cross-section of the cotton seed used in this work are presented in the supporting information section (figure S1 (available online at stacks.iop.org/JPD/54/325205/mmedia)).

2.2. Plasma reactors and treatment conditions

Most cold-air atmospheric-pressure plasma treatments were carried out in a dielectric barrier discharge (DBD) stainless-steel reactor with a flat-parallel electrode configuration, as represented in figure S2(a). The setup consisted of two parallel circular metal electrodes 80 mm in diameter with upper and bottom metal griddles 13 mm and 9 mm thick, respectively. A quartz dielectric round plate (dielectric constant $\varepsilon = 5$) 0.5 mm thick and 100 mm in diameter was stuck to each metal electrode using silver paint. The larger diameter of these dielectric plates vs. that of the metal electrodes prevented the production of micro-discharges by edge effects. The gap between electrodes was adjusted with a screw meter attached to the upper electrode (with a 1.4 mm thread pitch). A typical distance of 5.6 mm was used for the seed treatments. An AC electrical voltage was generated using a function generator (Stanford Research System, Model DS345, Synthesized Function Generator) connected to a high-voltage amplifier generator (Trek, Model PD05034, High Voltage Amplifier) applied to the upper electrode. Air plasmas were generated at two pressures (350 and 500 mbar), producing filamentary discharges, which were, in part, localized on the seeds. A peak-to-peak voltage of 16.4 kV and a frequency of 1 kHz were chosen as optimal conditions for seed activation. Seeds (30 seeds at each time, placed on the bottom electrode of the reactor) were treated under these conditions for a period of 15 min. Averaged peak-to-peak current values of 6.6 mA and 3.8 mA were measured at the two working pressures, corresponding to applied power values of 3.2 W and 1 W, respectively, as shown in figure S2 through their corresponding $V(t)$ and $I(t)$ representations. These measurements were performed with a 223 Ω resistor in series with the reactor and the ground and confirmed using a 6585 Pearson current monitor with a 1 ns response time. As reported in the results section, these conditions produced little alteration in the surface morphology of the seed bark.

An alternative set of treatments was carried out in a portable glass reactor consisting of two parallel metal electrodes (45 mm in diameter) that were protected by glass dielectric plates separated by a gap of 12 mm. These treatments aimed at studying the D_2O uptake capacity of pristine and plasma-treated seeds using infrared spectroscopy. The reactor was fed with a helium/air flow mixture at 5 $l_n\,min^{-1}$, a voltage of 20 kV at 16 kHz, and 30 W of applied power for 2 min of treatment (see [19, 21] for more details). The treatment time and experimental conditions ensured that the seeds had an equivalent wetting behavior and similar surface morphological aspect and composition to the seeds treated in the first reactor, as determined by SEM and FT-IR. The use of this portable reactor ensured that the exposure time of seeds to the air prior to their FT-IR analysis or related water vapor exposure experiments was shorter than 1 min. (see section 2.3).

2.3. Characterization

Plasma-treated seeds were analyzed shortly after surface treatment for the characterization, germination and sterilization tests. However, in the case of XPS, the seeds had to stay in the vacuum chamber for a minimum of 24 h to reach the pressure required for analysis.

Optical emission spectroscopy (OES) characterization of air plasmas was carried out during the plasma activation treatment. Spectra were collected using an optical fiber connected to a monochromator (Jobin-Yvon FHR640) with a spectral resolution and an integration time of 0.2 mm and 0.5 s, respectively (see figure S2(a)). A diffraction grid with 1200 lines cm^{-1} and entrance and output slits of 1 mm was used to collect the spectra. Signals were recorded using a photomultiplier working in the wavelength range from 200 to 900 nm.

The wetting behavior of plasma-treated and pristine cotton seeds was examined by dripping 50 μl water onto their surfaces. The water uptake experiments consisted of the exposure of seeds to saturated water vapor for a given period of time (∼100% relative humidity with either H_2O or D_2O) in a closed box at 21°. For this purpose, 20 seeds were placed into a dry Petri dish floating on the liquid water. Water absorption tests consisting of seed immersion in liquid water for given periods

of time were also carried out. These two experiments provided information about the initial steps of water uptake capacity, determining their influence in germination. Three water uptake experiments were performed in each case and the reported data are averaged over the three sets of obtained results. The seeds were weighed before and after the water uptake with a precision balance (±0.01 mg). The water uptake, determined after water vapor exposure or water liquid immersion for different times, is expressed in terms of weight percentages with respect to the initial weight of 60 seeds. In order to assess the effect of the plasma treatment on seeds subjected to adverse conditions, the immersion experiments in liquid water were limited to 30 min. For longer times, the plasma-treated bark swelled and became detached from the seed core. Weighing was carried out after placing the seeds onto blotting paper in ambient air for a few seconds to remove unabsorbed liquid water.

D_2O vapor uptake experiments were done in the same hermetic container described above filled with D_2O (∼100 R.H. y ∼21 °C) and with 20 seeds located in the Petri dish floating on the liquid. To systematize the FT-IR analysis after each D_2O vapor uptake test, the seeds were removed from the container after the selected exposure time; then, they were weighed in less than 1 min and subsequently brought to the FT-IR spectrometer, where the spectra were recorded within less than 5 min. Three replicated weight measurements and FT-IR analysis were performed for each D_2O exposure.

The SEM analysis of seed surface topography, including cross-sectional images, was performed without metallization using a Hitachi S4800 SEM-FEG field emission microscope working at 2 kV. Elementary and compositional maps were obtained using an x-ray detector (EDX) (an EDX-Bruker-X Flash-4010 analyzer) working at 20 kV.

The chemical state of the cotton surface was characterized by XPS using non-monochromatic Mg Kα radiation ($h\nu$ = 1253.6 eV) and a hemispherical analyzer (SPECS, DLSEGD-Phoibos-Hsa3500), working in a constant-pass energy mode at a value of 50 eV for the general survey and 30 eV for highly resolved spectral zones. The spectra were calibrated using the binding energy (BE) of the main C1s peak at 284.5 eV attributed to C–C and/or C–H bonds [27]. The atomic concentrations of elements on the seed surfaces were determined from the areas of the peaks after subtracting the background and applying the sensitivity factors available for each element/photoelectron peak.

The FT-IR analysis of the cotton seeds before and after exposure to D_2O vapor was performed using a Nicolet AVATAR 360 spectrometer in the range of 400–4000 cm^{-1}. Measurements were carried out using a Smart iTR attenuated total reflectance (ATR) sampling accessory (Thermo Scientific Inc., U.S.A.). Spectra were obtained with an average of 32 scans and a resolution of 4 cm^{-1}. An advanced ATR correction algorithm (OMNIC 7.3 from Thermo Electron Corporation) was employed to correct the band intensity distortion, peak shifts, and polarization effects.

2.4. Germination and contamination tests

Two culture tests were carried out to determine germination rates. These were expressed as the number of days elapsed after sowing in the following two media:

(a) *In vitro* germination and contamination in Murashige and Skoog (MS) liquid culture medium (Sigma Aldrich) without sterilizing treatment (referred as Cont./MS). This medium has a known composition [28] resulting from stock solutions of nitrates (KNO_3 and NH_4NO_3), sulfates, chlorides, phosphorus, boron and molybdenum.
(b) Potted germination (referred to as peat): seeds were buried in a pot of (Florabella) murky blond sphagnum mixed with black peat and also in frozen sphagnum and clay (pH 5.5–6.5, electrical conductivity 40 mS m^{-1} with added fertilizer NPK 14:16:18 in 1.5 kg m^{-3}).

The elapsed time before the appearance of the first radicle was taken as the germination time. The radicle growth, characterized by stem development and accompanied by leaf buds, was also qualitatively followed. Seeds sown in the peats were typically irrigated with 50 cc water. Drought conditions in peat tests were simulated using a sandier crop soil (texture: 71.3% coarse sand, 5.9% fine sand, 11.9% silt and 10.8 clay—classified as being in the textural class: sandy-frank). In this case, a first irrigation with 20 cc of water was done just after sowing, followed by 10 cc irrigation the next day.

Contamination tests consisted of the visual observation of the development of fungus or bacteria on seeds in the MS media. The appearance of a reddish stain served as the identification criteria for contamination. The germination potential (taken as the percentage of germinated seeds under the selected growth conditions) and the seed contamination rate are shown as bar charts with an average error of 5% in the statistics.

3. Results and discussion

3.1. Plasma discharge characterization

Figure 1 presents selected results for the spectroscopic characterization of the plasma discharge. Spectra were collected laterally from the reactor wall when the plasma was turned on in the presence of cotton seeds at air pressures of 350 mbar and 500 mbar. These spectra, unnormalized in intensity to compare the effect of pressure, are compared with a spectrum recorded from a plasma discharge at 350 mbar without any seed inside the reactor (note that plasma does not ignite in the absence of seeds at 500 mbar). Furthermore, figure S2 reflects the effect of seeds on the plasma discharge parameters: at 350 mbar, less intense filaments are observed when seeds are present on the grounded electrode whereas at high pressure, the presence of cotton seeds located on the grounded electrode enables the ignition of the plasma discharge because this effectively reduced the gap between the electrodes. Without seeds, 3.5 W and 0.2 W of applied power were measured for

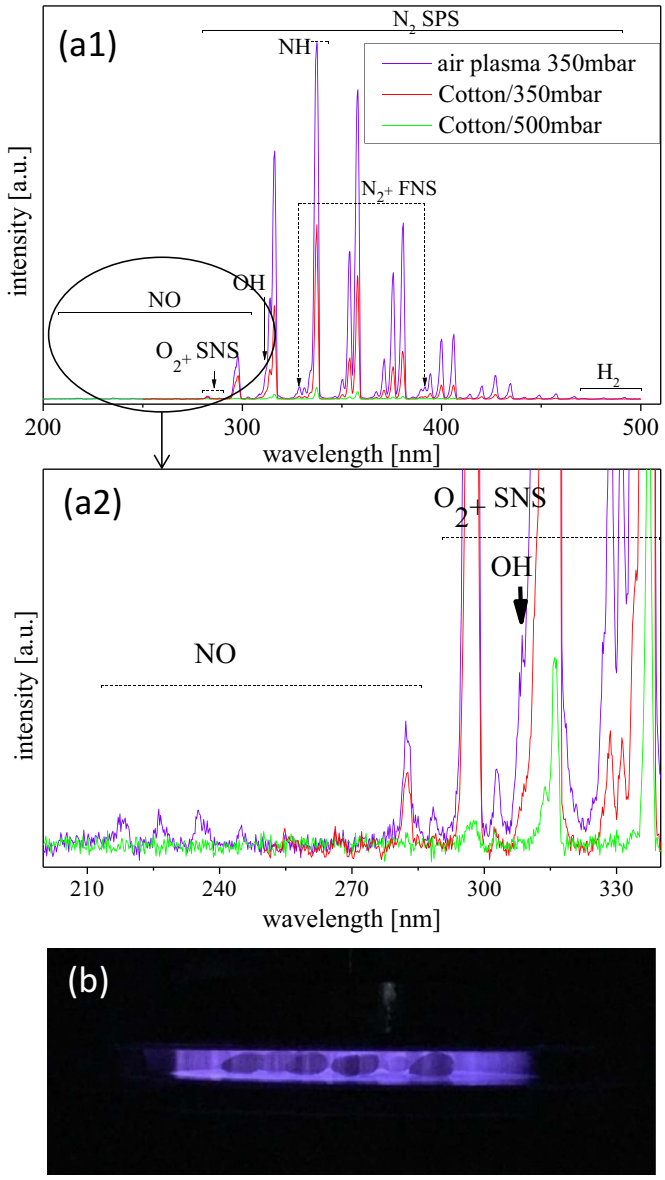


Figure 1. (a1) Optical emission spectra recorded during the air plasma seed treatments at 350 mbar and 500 mbar. A spectrum at 350 mbar without seeds in the reactor is also included for comparison; (a2) enlargement of the low-wavelength region of the spectra reveals the formation of NO* and OH* species in the plasma discharge; (b) image of the air plasma discharges generated in the presence of cotton seeds.

the 350 mbar and 500 mbar conditions, respectively; the latter is just the contribution through a capacitor. However, as mentioned in the experimental description, with seeds inside the plasma reactor, 3.2 W and 1 W of applied power were determined, respectively. From the OES spectra in figure 1, we attribute the overall intensity decrease observed in the presence of seeds to the highly filamentary nature of the plasma discharge that developed in this case (see figure 1(b)) and to the fact that a decrease in light collection capacity may occur if the sight line direction of the light-collecting fiber encounters a seed. When the pressure was increased, air plasma filaments were less numerous and appeared more separated, a feature that likely contributed to the observed decrease in peak emission intensity (figures 1(a1) and (a2)). However, due to the low applied power in both at high-pressure conditions (lower than 3 W), overheating effects can be discounted despite the filamentary nature of the plasma, as previously determined for the same plasma reactor [20]. Nevertheless, the possibility that an effective reaction of activated plasma species with the surface of seeds may contribute to a certain depletion in the plasma discharge cannot be ignored. However, most relevant for the present work is that equivalent spectral bands (i.e., appearing at the same wavelengths) were observed in the three cases.

The recorded spectra showed that molecular bands associated with excited nitrogen molecules presented the highest intensity as compared with the bands of other excited species. These molecular bands include the transition lines corresponding to the first negative system of the molecular ion N^+_2 [$B^2\Sigma_{u+} \rightarrow X^2\Sigma_{g+}$] at 391.4 nm and to the second positive SPS system of the excited neutral molecule N_2* [$C^3\Pi \rightarrow B^3\Pi$] in the range of 290–385 nm [1]. The emission peak of this system at around 337 nm would also be compatible with the formation of NH* species (i.e. NH molecular band [$A^3\Pi \rightarrow X^3\Sigma$] [29]) that, even if unlikely, might form due to the interaction of excited species of nitrogen with bonded hydrogen atoms at the surface of the seed bark. In the ultraviolet region, NO* radicals can be identified by their characteristic γ system [$A^2\Sigma^+ \rightarrow X^2\Pi$] [2], while the bands of the O_2^+ second SNS negative system [$A_2{}^2\Pi \rightarrow X_2{}^2\Pi$] can be also observed in the spectra of figure 1(a2) [30].

Overlapping with these lines, there are also some hints of a OH* band at 309.0 nm. OH* species may originate from residual water present in the atmosphere or even stem from the cotton seed. These species, or those resulting from their reaction with the seed surfaces, can lead to the generation of other ROS and RONS. Effectively, oxygen and nitrogen excited species, together with hydroxyl radicals, can act as very effective plasma functionalizing and etching agents of the bark's surface, giving rise to the various chemical and surface morphological changes reported below. Effective sterilization may be another consequence of this air-plasma/seed interaction.

3.2. *Surface morphology of plasma-activated cotton seeds*

Previous studies of the plasma activation of cotton seeds have reported that effective plasma etching affects seed hydrophilicity and may improve water permeation up to the endosperm and embryo [5]. In comparison, plasma-activation treatments utilized in the present work were relatively mild, because highly damaged seed coats do not provide long-term protection for storage, handling and planting, and these seed uses are more susceptible to bacterial contamination. As reported below in section 3.5, the treatments used here were beneficial in improving both sterilization and germination capacity; the latter is most likely associated with the surface formation of specific functional groups. A question for debate is whether these functional groups may act as RONS [17, 18] and/or include peroxide-like species which are well known for deactivating the germination-retardant ABA hormone [31, 32].

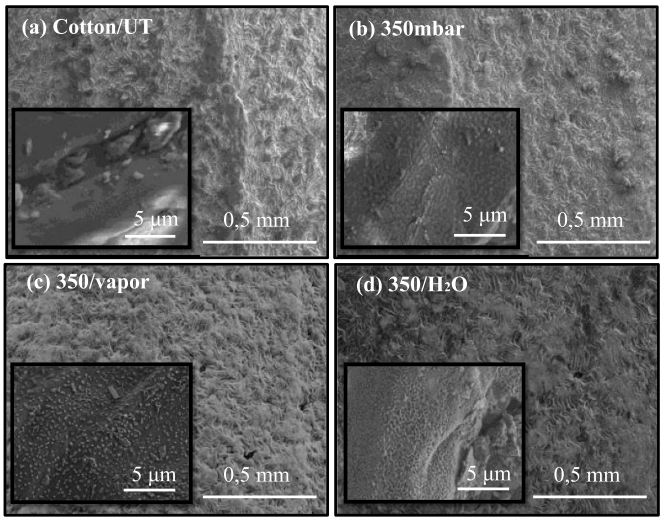


Figure 2. SEM images at two magnifications of: (a) the pristine surface of a cotton seed; (b) the state achieved after plasma activation for 15 min at 350 mbar of pressure; (c) after a subsequent exposure to water vapor for 1 h or (d) after immersion in liquid water for 30 min.

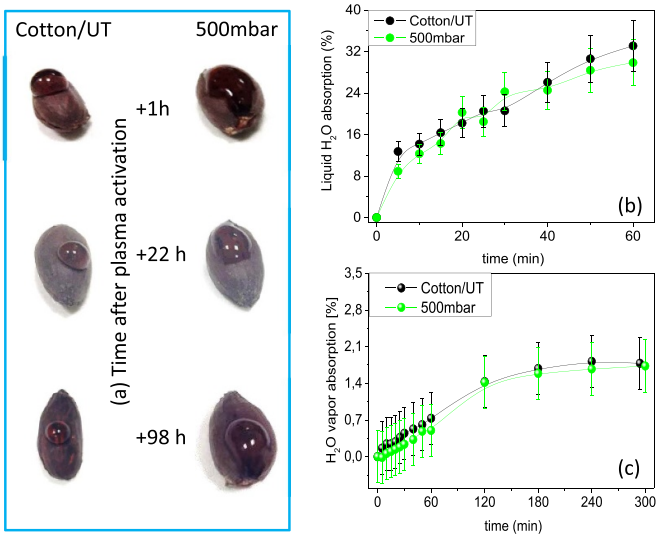


Figure 3. (a) Photographs of pristine (UT) and plasma-treated seeds (500 mbar) after deposition of a drop of water of 50 μl to determine the hydrophobic/hydrophilic character of the surface. Photographs are shown for seeds top) 1 h, middle) 22 h and bottom) 98 h after plasma treatment; (b) water uptake capacity from the liquid phase for the pristine and plasma-treated seeds (500 mbar) for increasing periods of time; (c) water uptake capacity from the vapor phase for the plasma-treated seeds (500 mbar) compared to the pristine ones.

The plasma treatment used here slightly affected the seed surface morphology, as evidenced in figure 2. SEM micrographs were also taken after the exposure of the plasma-treated seeds to water vapor or after their immersion in liquid water. The original surface of seeds (cf, figure 2(a)) exhibits a rough heterogeneous aspect, on which grooves and asperities can be discerned. The plasma treatment (figure 2(b)) seems to be efficient in removing some of these asperities, leading to a more homogeneous surface. According to figure 2(c), exposure to water vapor for 1 h induces the development of small grains on the otherwise homogeneously rough surface. However, when the seeds were immersed in water for half an hour (the maximum time before some seeds began to open their bark and the radicle started to emerge), the opposite happened: holes or craters replaced the grains (see figure 2(d)). We hypothesize that the grains are fat accumulations or etched fragments of the surface crust that agglomerate in the form of grains favored by the water layer formed upon water adsorption on the plasma-treated surface (see section 3.5). These accumulations or fragments, once formed, seem to be removed or dissolved by liquid water, thus leaving the hollow surface found after immersion. To account for some of the experimental results found in this investigation, it is relevant that the distribution of elements such as K, N, Ca and Cl in the interior of pristine seeds, determined by EDX during their SEM characterization, was rather homogenous, with just a little enrichment of potassium in the bark zones (figure S1 in the supporting information) that is blurred after their interaction with water (figures S3 and S4).

3.3. Water interaction with plasma-treated seeds

It is widely accepted that the plasma treatment of seeds induces a hydrophilic surface state [33, 34] and causes them to favor the interaction with, and the uptake of, water [35]. The plasma treatments performed in this work effectively transformed the surface of cotton seeds from hydrophobic to hydrophilic. Figure 3(a) presents a series of photographs showing that, just after treatment, or even for several days afterward, a water droplet spreads on the plasma-treated seeds and covers a larger area than on the untreated seed, where it develops a spherical shape. The hydrophilic state of the seeds that were plasma treated at these low applied power values lasted for more than one month. This evidence supports the long-lasting effect of the plasma treatments, which would be compatible with the large-scale handling of seeds, as required for industrial processes and applications. In agreement with previous works, we attribute this effect to the preferential etching removal of the wax component of the bark and/or to surface functionalization with polar groups [17, 18].

Unlike various works in the literature reporting that plasma seed activation strongly enhances the water absorption capacity from the liquid phase [34–36], and that this can be correlated to the induced hydrophilic state, immersion experiments in liquid water carried out in this work showed a rather similar water uptake capacity for the pristine and plasma-treated seeds at 500 mbar (cf figure 3(b)). On the other hand, as reported in figure 3(c), the water uptake from the vapor phase was much less than from liquid water, with a small difference between plasma-treated and untreated seeds.

The water vapor experiment was carried out for a maximum exposure time of 6 h, after which, the uptake capacity seemed to reach a steady state (figure 3(c)), and the seeds are believed to activate other respiration and metabolic activation processes. This plateau-like behavior agrees with a previous observation by Nonogaki *et al* [37], which showed that 4/5 h

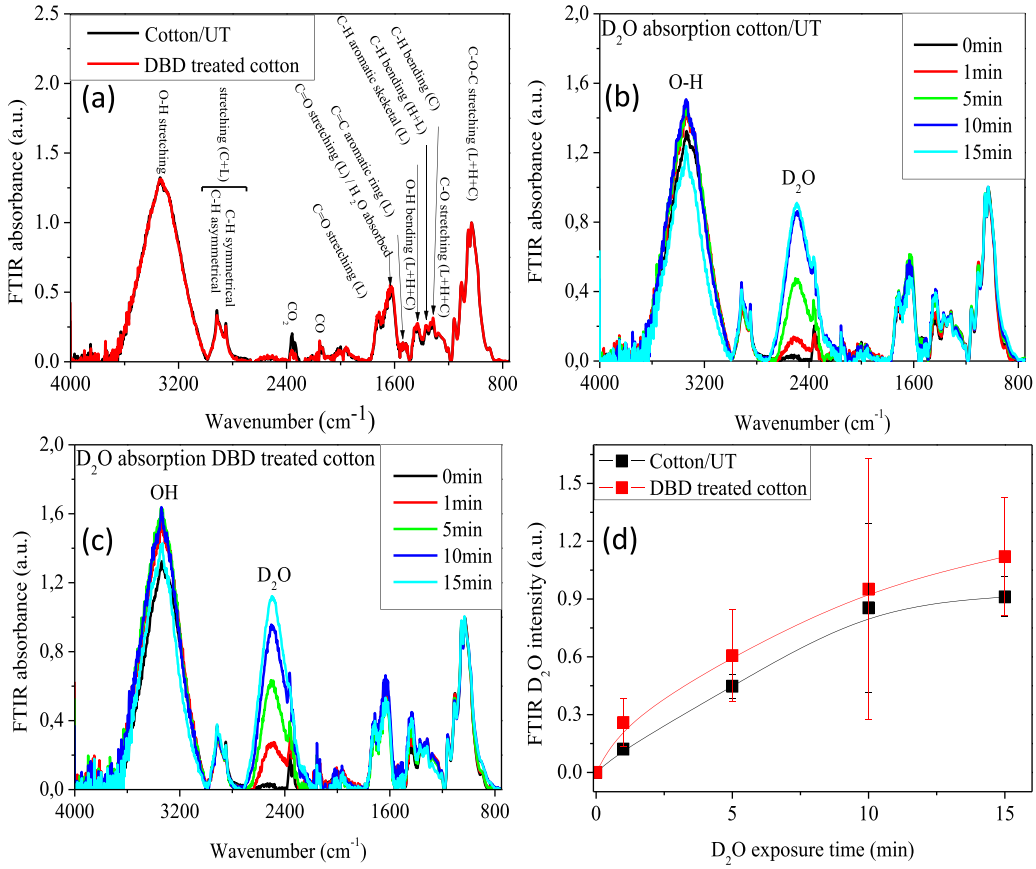


Figure 4. FT-IR analysis of the surface of cotton seeds: (a) before and after the air plasma treatment in the portable reactor. C: cellulose, H: hemicellulose, L: lignin; (b) and (c) FT-IR bands' evolution as a function of exposure time to D_2O vapor for untreated and plasma-treated cotton seeds, respectively; (d) plot of the FTIR D_2O band intensity (2493 cm^{-1}) versus the D_2O vapor exposure time for the pristine and DBD plasma-treated cotton seeds.

of water contact is enough for complete imbibition before triggering the germination process. After six hours, the slope of the water absorption curve increased again (data not included in figure 3), very likely because of the mobilization of the stored reserves that, becoming accessible to water, trigger the radicle emergence. In comparison with treatments carried out by other authors [5, 38, 39] that led to the formation of severe surface cracks and cellulose degradation, the less aggressive character of the plasma treatments in this work might be the reason why water uptake is not particularly promoted by our plasma experiments.

In order to gain a deeper insight into the seed surface/water vapor interaction, the initial stages of water uptake into the outermost zone of the bark were investigated by FT-IR analysis of the seed surface for the pristine and plasma-treated states exposed to D_2O vapor (note that the thickness probed by this technique is around 1–2 μm). The results of this experiment are reported in figure 4 for various exposure times, up to a maximum of 15 min.

Figure 4(a) shows the FT-IR spectrum recorded for the untreated seeds, including the accepted attributions of their main bands and peaks. The vibrational modes at around 3600–3200 cm^{-1} are attributed to the stretching vibration of −OH functional groups and water present in the seed's surface. The bands at ∼2900 cm^{-1} and ∼2850 cm^{-1} are due to C–H vibrations, most likely due to cellulose (C), hemicellulose (H) and lignin (L) components [21]. Meanwhile, C = O stretching bands of the carboxylic acid or ester and carboxylate groups can be seen in the 1750–1650 cm^{-1} and 1650–1630 cm^{-1} regions, respectively [39]. The bands at around 1730–1500 cm^{-1} and 1500–1180 cm^{-1} may stem from the contributions of molecular H_2O and –OH/C–H groups, linked to wax, pectin and hemicellulose (H) signals [40]. The spectral bands appearing between 1500 and 1180 cm^{-1} can be attributed to C–H bending and aromatic modes, while those located at 1180–840 cm^{-1} may correspond to C–O–C stretching modes. According to Wang *et al.* [5], the bands below 1500 cm^{-1} account for cellulose (C) bond vibrations (cellulose is a major component of the cotton seed coat), C–H bending bands for sugars, crystalline regions of starch and pectinand the C–O–C pyranose ring skeletal vibrations, among others. No significant changes can be distinguished in the FT-IR spectrum (figure 4(a)) after plasma treatment. However, D_2O incorporation into the seeds upon exposure to vapor of deuterated water would clearly be evidenced by this technique. The spectra in figures 4(b) and (c), normalized to the C–O band at 1029 cm^{-1}, were taken after exposure of the pristine and the plasma-treated seeds to D_2O

vapor, respectively. These two series of spectra show the development of a broad band centered at 2493 cm^{-1} attributed to the stretching vibration of the O–D bond in the molecular D_2O incorporated in the outermost layers probed by the FT-IR technique [41]. From the spectra in figures 4(b) and (c) it is possible to estimate the amount of deuterated water incorporated in the outer zone as proved by FT-IR spectroscopy. We stress that the water uptake capacity could hardly be distinguished using H_2O instead of D_2O vapor, because the new contribution due to adsorption would sum up to the bands that already exist at around 3600 and 1600 cm^{-1}. The analysis of the evolution of the D–O stretching band as a function of exposure time in figure 4(d) shows that, within the range of experimental error, the intensity of the D_2O band was slightly higher for the plasma-treated seed. For exposure times longer than 15 min, the intensity of the monitored band progressed very little in the two cases (a maximum intensity was found after 30 min exposure), suggesting a certain water saturation of the outer bark zone examined by FT-IR. Interestingly, for exposure times up to 10 min, the increase in the D_2O band intensity was accompanied by a small increase in the H_2O/OH-bands at 3600/1600 cm^{-1}, suggesting that an outward diffusion of H_2O from the interior of the seeds and/or bark was taking place in parallel with the massive incorporation of D_2O. This effect proved the presence of an effective mobilization and exchange of water through the seed bark.

When plotting the intensity of the IR band due to D_2O/-DO against the amount of absorbed water determined gravimetrically in figure 3(b) (cf. figure S5(a)), one obtains a direct correlation that supports the use of the FT-IR analysis to follow the initial water uptake capacity of seeds. A closer look to this plot, however, shows that while the gravimetrically estimated water uptake is slightly higher for the untreated than for the plasma-treated seed, its estimation from the intensity of the D_2O FT-IR band presents the reverse trend, where the plasma-treated seed depicts a faster evolution for the water incorporated in the outer zones of the bark examined by this technique. This apparent contradiction can be reconciled if we assume that, in the initial stages, water remains preferentially confined in the outer zones of the plasma-treated seeds whose surface state has been transformed into hydrophilic (see the scheme in the figure S5(b)). However, we should emphasize that these slight initial differences in surface water uptake do not contradict the fact that the overall water uptake capacity from the vapor phase is rather similar in the pristine and plasma-treated seed (cf. figure 3).

3.4. Germination and sterilization assays after plasma activation

The germination potential was determined by sowing the seeds in both the MS medium and in the peat, as reported in the experimental section. Figure 5 presents the results of these tests, together with those of the sterilization ratios obtained for the pristine and plasma-treated seeds.

After seven days of culture in peat, the germination potential of the plasma-activated seeds either at 350 (100% germination potential) or 500 (90% germination potential) mbar was higher than for the untreated seeds (80% germination potential). Similar tendencies have been found in previous works [5, 16], where DBD plasma treatments were more intense, in terms of both time and applied voltage, than in our case. Meanwhile, figure 5(b) shows equivalent results for germination in MS culture media: both untreated and all plasma-treated seeds presented a 100% germination potential after six days. Remarkably, the contamination degree of the untreated seeds reached a value of 20% following the second planting day (at the end of the enzymatic activation stage, when the radicle begins to emerge) whereas the plasma-activated seeds remained completely sterilized, even after seven days or longer.

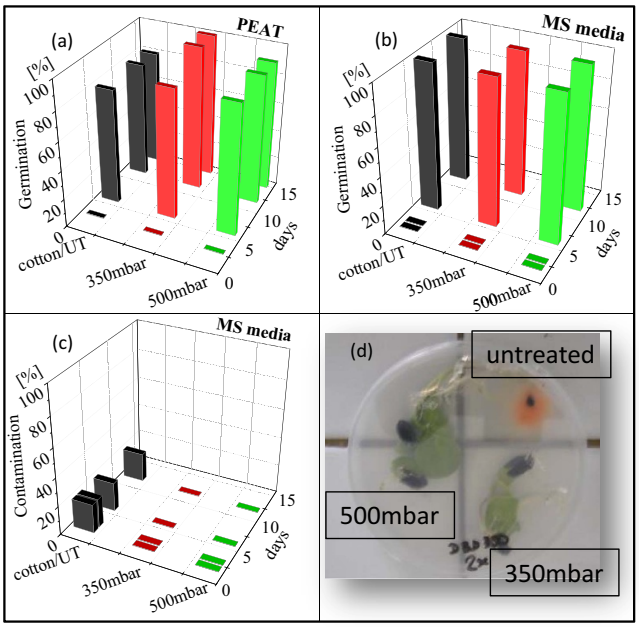
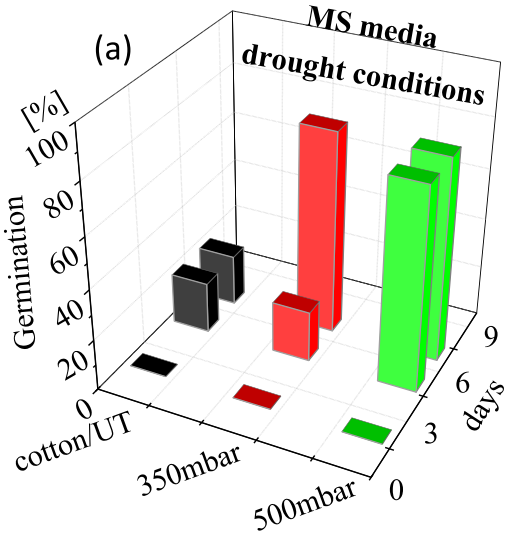


Figure 5. Germination potentials expressed as percentages of germinated cotton seeds after plasma activation at two different pressures, compared with that of untreated seeds in peat (a) and MS media (b); (c) seed contamination diagrams for untreated and plasma-treated seeds at the two selected pressures; (d) picture of the seed contamination assay for the three examined conditions.

A photograph of the contamination test is presented in figure 5(d), where the reddish coloration in the MS media is a sign of fungal or bacterial contamination. Effective sterilization is a known effect of nitrogen-rich high-pressure plasmas, which has been attributed to the effect of UV radiation [42], likely through the rupture of the microbial genetic material [43]. It is known that energetic UV photons speed up the germination process due to the breakdown of seed coatings, although they have other associated effects such as retarded seedling growth and root damage [44, 45]. However, under our working conditions, UV photons of low intensity are mainly associated with the weak emission lines due to the second positive system of activated N_2^* species (figure 1) and, consequently, a substantial effect on germination can be discarded. Also, RONS generated by plasma at the seed surface might be effective for sterilization [46] because they may



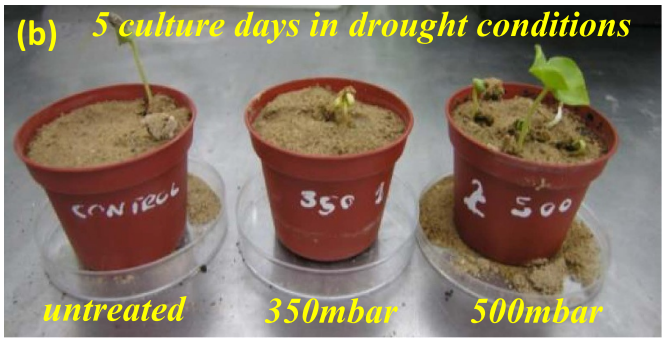


Figure 6. (a) Germination potential as a function of the number of culture days in peat under drought conditions; (b) picture of the plants grown after five days of culture in a dry soil from the untreated and plasma-treated seeds at 350 mbar and 500 mbar.

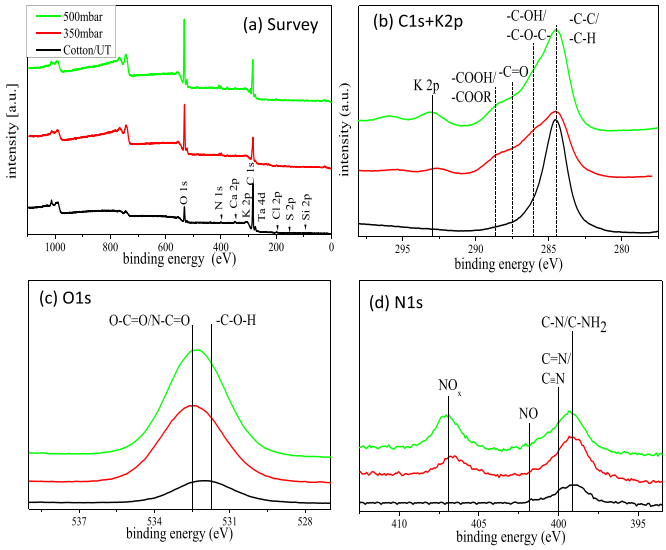


Figure 7. XPS general survey spectra (a) and high-energy resolved regions of carbon + potassium (b) (C1s + K2p), oxygen (c) (O1s) and nitrogen (d) (N1s) zones recorded for the untreated and plasma-treated seeds under two pressure conditions.

promote specific pathogenic responses, including cell death through oxidative stress processes [2, 4, 47].

Although cotton seeds may absorb moisture and germinate in relatively dry soils [48], the germination rate and ratio can be seriously affected by the degree of humidity of the medium. Herein, we investigate the effect of the plasma treatment on germination under water scarcity conditions. Figure 6(a) shows that after five days of culture (i.e. in the growth stage just at the moment when the plant usually begins to emerge), 80% of the cotton seeds treated with plasma at the highest pressure had already germinated, while only 20% germination was found for untreated and lower-pressure plasma-treated seeds. The outstanding result was that after seven days, the seeds treated under both plasma pressure conditions presented a germination potential of 80% while the germination of untreated seeds remained at a low level of 20%. It is also noteworthy that, according to the images in figure 6(b), not only the germination rate but also the robustness of the emerging plant was favored by the plasma treatment at the highest pressure. These differences stress the potential of plasma to enable germination under unfavorable climate conditions.

These large differences in germination capacity beg the question about the factor(s) producing this enhanced germination in plasma-treated seeds. Traditionally, an increase in the water uptake capacity has been claimed to account for the improvement in the germination of plasma-treated seeds [36, 49]. However, our results here, without discounting this as a beneficial factor, suggest that additional effects must be involved in promoting the germination capacity of seeds. To try to shed some light on this question, we propose that, despite the differences, both the drought conditions used in the peat sowing experiment (cf. figure 6) and the water vapor exposure tests affected the surface state of seeds after plasma treatment at the two pressures similarly. We will show next that the utilized water vapor exposure conditions enable a controlled investigation of the chemical state of the surface and its relation to the observed differences in germination.

3.5. *Chemical state of seeds: surface analysis*

Changes in the surface morphology, hydrophilicity and water uptake capacity (cf. figures 2–4) of the plasma-treated seeds were accompanied by significant changes in surface composition, as revealed by XPS analysis. This technique monitors approximately 3–4 nm of the sample's depth. Figure 7 shows results for untreated and plasma-treated seeds under the two plasma conditions utilized in this work. This figure gathers general and zone spectra of the C1s + K2p, O1s and N1s levels, while table 2 shows the percentages of carbon, oxygen, nitrogen, and potassium determined for the pristine and plasma-treated seeds. This table also includes results after seed exposure to water vapor and liquid water immersion that will be discussed below in more detail.

This table reveals that after plasma treatment, the carbon content at the surface decreases by 25%–30%, the oxygen concentration increases by around 22% and that of nitrogen increases by approximately 1% (up to ca. 2.5%). Interestingly, a small but measurable amount of potassium appears at

Table 2. Atomic composition by XPS analysis of seeds after different treatments including pristine (UT) and plasma-treated seeds at two pressures. Equivalent data for pristine (UT) and plasma-treated seeds at the highest pressure after their exposure to water vapor or immersion in liquid water

Atomic composition (%)	[C]	[O]	[K]	[N]
Cotton/UT	84,0	11,2	0,0	1,5
350 mbar	54,5	33,6	0,3	2,6
500 mbar	59,4	33,5	1,3	2,4
UT/1 h vapor	81,1	15,3	0,0	1,6
500 mbar/1 h vapor	69,3	23,4	0,1	2,7
UT/30 min liq. H_2O	78,5	15,4	0,2	2,8
500 mbar/30 min liq. H_2O	71,3	22,4	0,0	3,0

the surface after plasma treatment, particularly after the treatment at 500 mbar. The set of C1s spectra in figure 7(b) shows that the decrease in carbon content entails the loss of C–C/C–H groups (E_B = 284.5 eV), which are majority in the seed bark and must be responsible for the hydrophobic behavior of pristine seeds [5]. According to this figure, the changes in atomic percentages gathered in table 2 are accompanied by a substantial change in the chemical characteristics of the functional groups detected at the seed surface. After plasma treatment, particularly for treatment at the highest pressure, the C1s spectrum reveals the formation of new groups associated with the observed increase in oxygen that appear in the form of new –CO_x functional groups (C1s shoulders/bands due to –C–OH (E_B = 286.0 eV) and –C = O and –COO^- (E_B = 287.5 and 288.6 eV [43]). The increase in the oxygen percentage (cf. Table 2) and the shift to higher BE values observed in the O1s band agree with the formation of these or similar surface groups. Unfortunately, the O1s spectra are characterized in all cases by a single broad band (full width at half maximum, FWHM, of approximately 3 eV), in which no defined peaks or shoulders can be discerned, although the observed shift of the envelope band to higher BE values indicates the formation of functional groups containing covalently bonded oxygen atoms. It is also noteworthy that, in figure 7(b), the 350 mbar plasma seems to generate more oxygenated C–OH groups (maximum at E_B = 531.7 eV), whereas at 500 mbar the treated surface seems to incorporate relatively more O = C–O and COO– (E_B = 532.5 eV) species. The formation of oxygenated carbon functional groups after plasma treatment must be responsible for the transformation of the surface into a hydrophilic state. We also hypothesize that plasma treatment may also induce the formation of adsorbed peroxide-like species, which may act as effective ROS in the germination process [10]. Although not identifiable in the unstructured O1s spectra, its large width and the shift to higher BEs would be compatible with the formation of these peroxide-like species [49, 50]. In γ-irradiated polymers, the formation of similar species has been proposed as responsible for triggering their oxidative degradation [51].

The XPS analysis of the untreated seed also revealed the existence of nitrogen species characterized by a peak at E_B = 399.2 eV (figure 7(d)) that must correspond to C–N/C–NH_2 functional groups present in the macromolecules of the bark membrane [52]. The intensity of this peak, with its maximum slightly shifted to approximately 400.1 eV, increased after plasma activation, while a new feature at around 406.0 eV also developed after these treatments (figure 7(d)). These new spectral features can be attributed to the formation of –N–O (E_B = 401.8 eV) and –NO_x (E_B = 406.0 eV) functional groups [53]. We claim that these new surface species are formed because of the interaction of the seed surface with the N_2^+ ions or N_2^* species and/or the reaction with oxygen excited species and OH^*. Plasma oxygenated species can also induce the formation of –NO and –NO_x species by reaction with C–N(H) surface groups [53]. –N–O and –NO_x surface groups can also form through the surface adsorption/reaction of the NO^* radicals detected by OES in the plasma. In addition to the possible formation of peroxide-like species acting as ROS [54, 55], we postulate that some of these –NO/–NO_x adsorbed species might act as effective RNSs, thereby contributing to the acceleration and enhancement of the germination capacity of the seeds [46]. The function of these RONS would be similar to that of H_2O_2 that, at low concentrations, favors seed germination because it reacts with the endogenous ABA, a specific metabolic enzyme responsible for breaking seed dormancy [6, 56].

In the spectra in figure 7, it is also remarkable that potassium, in the form of K^+ species (K $2p_{3/2}$ E_B = 293.3 eV), appears at the seed surface after plasma treatment (figure 7(b)). Plasma treatment seems to induce the surface segregation of potassium ions by diffusion from the interior of the seed bark, where a rather homogeneous distribution of this element was determined by EDX analysis (see supporting information figure S1). In a previous work with quinoa seeds [20], we found a similar surface migration of potassium upon plasma activation. Although still requiring a specific investigation, we suggest that the mobilization of potassium toward the surface (and back upon exposure to water, as we will discuss next) can also be important to trigger the germination process. In fact, this element seems to regulate the physiological processes of carbon and nitrogen exchange between seeds and soil [57]. Moreover, cotton crops are known to be quite sensitive to a lack of potassium, even in soils that were not considered potassium deficient *a priori* [58].

Another interesting piece of evidence provided by the XPS analysis is the observation that the distribution of functional groups at the surface drastically changed when the seeds were exposed to water vapor or immersed in liquid water. The XPS spectra in figure 8 and the data in table 2, corresponding to pristine and plasma-treated seeds at 500 mbar, show clear evidence of the disappearance from the surface of potassium ions, a considerable number of oxygenated groups, and the NO_x species. We attribute these changes to the migration of these species to the interior of seeds, where they cannot be detected by this technique (note that upon exposure to water vapor, no dissolution in the liquid phase can be claimed to explain the disappearance of these species from the surface). This migration process was accompanied by surface composition

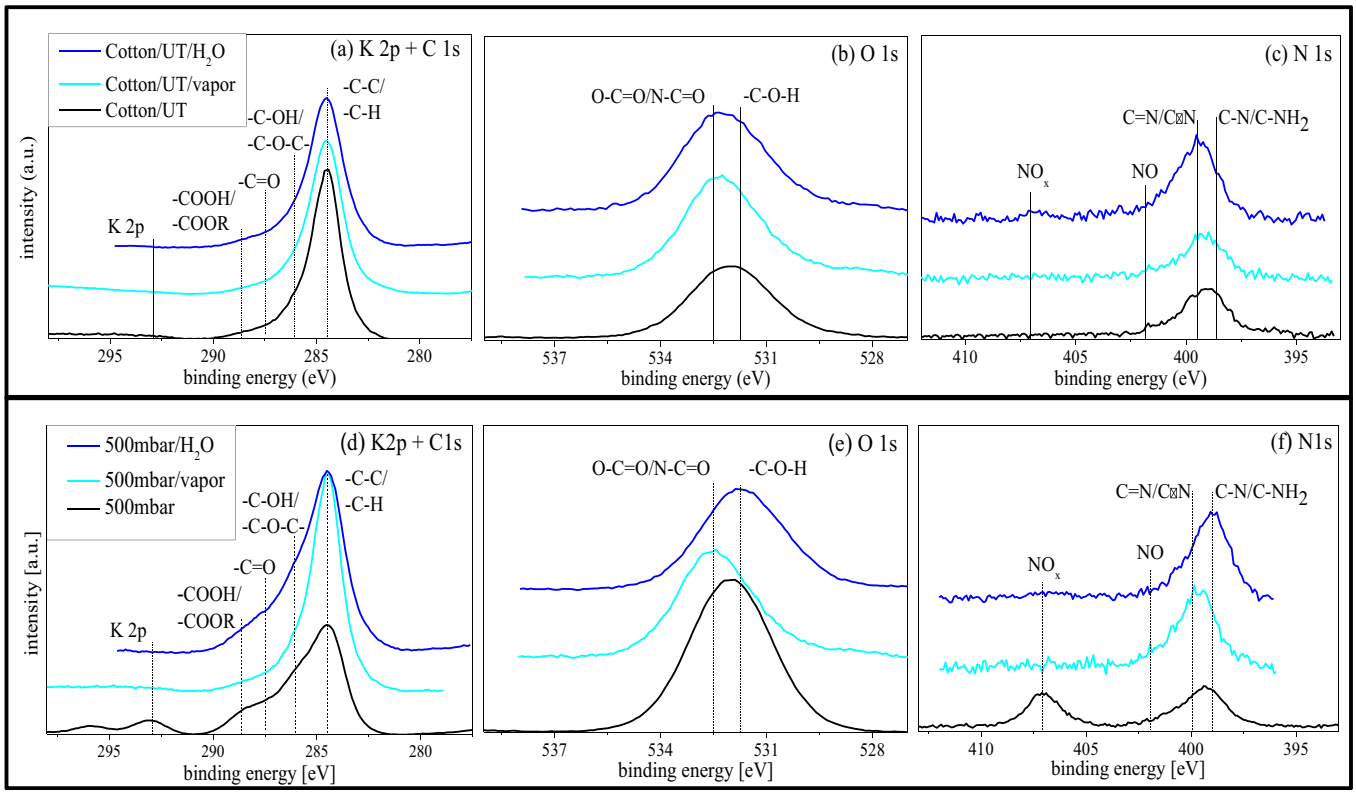


Figure 8. High-energy resolved spectra of carbon C1s, oxygen O1s, and nitrogen N1s for the untreated seeds ((a)–(c)) compared to the plasma-treated seeds at 500 mbar ((d)–(f)) when they were exposed to water vapor for 1 h or immersed in liquid water for 30 min.

changes characterized by an increase in the relative intensity of the C–H and C–C functional groups, which was particularly noticeable in the plasma-treated sample where the oxygen surface concentration also decreased with respect to the previous state. The different BE positions of the O1s envelope after water vapor exposure or water immersion can be accounted for by the preferential loss of $–COO^-$ groups (and perhaps peroxide-like species) after immersion in the liquid medium and the fact that the C1s shoulder due to –C–OH groups is still rather intense. It is noteworthy that the observed depletion of oxygen at the surface after contacting the plasma-treated samples with water does not contradict the fact that water is efficiently incorporated in the seed outer layers as evidenced by the gravimetric and FT-IR data in figures 3 and 4. This simply indicates that imbibed water is efficiently removed from the seeds during their conditioning in vacuum before the recording of the XPS spectra.

A similar behavior regarding the inward diffusion of species upon water exposure was observed in plasma-treated quinoa seeds [20], where the plasma also contributed to an improvement in their germination efficiency. We tentatively propose that some of the functional groups generated by plasma and removed from the surface upon water exposure can act as RONS factors, whose diffusion into the interiors of seeds would be responsible for the observed increase in germination potential [10, 57]. In this regard, it is interesting that no significant differences in XPS element distribution have been detected between the plasma-treated seeds exposed to water vapor or those immersed in liquid water (cf. figure 8 and table 2) and that, therefore, the inward diffusion of these species is likely to occur under both water-abundant and relatively drought conditions.

Although additional investigations are still required to clarify the role of RONS in germination, the previous results strongly support the position that the main factor affecting the germination of plasma-treated seeds is the generation of surface chemical groups that, upon water exposure, diffuse to the interior of seeds and contribute to the germination process [4, 10, 17, 18, 59, 60]. This diffusion process would not require abundant irrigation and, therefore, might be responsible for the observed enhancement of peat germination found under simulated drought conditions (cf. figure 6). This proposal, which accounts for the improvement of germination capacity upon plasma exposure, is still rather simple. For example, it does not consider the role that the oxygenated species might play once they diffuse into the interiors of seeds, where they could act as a swelling source, contribute to cellular respiration, react with glucose and provide energy for radicle emergence or inhibit the ABA signaling to retard germination [31, 32, 40]. In addition, even if, in the present case with cotton and mild plasma treatments, we have not found a significant increase in water uptake upon plasma treatment, this does not contradict the ample literature on the subject that shows that plasma contributes to an increase in water imbibition. Put simply, the results obtained here suggest that other factors are also involved in the improvement of germination resulting from seed treatment with plasmas.

4. Conclusions

High-pressure plasma activation of cotton seeds at low applied powers has been demonstrated to be a useful procedure for enhancing their germination potential, not only under the usual sowing conditions but particularly in drought environments. A drastic decrease in the seed contamination degree was another beneficial consequence of the atmospheric plasma treatment. A thorough analysis of the water uptake capacity before and after plasma treatment did not show significant differences in this feature for the plasma-treated seeds. In this regard, we demonstrate that tracking the evolution of FT-IR bands associated with deuterated water incorporated into the seeds can be a suitable methodology to follow the incorporation kinetics of water into the external layers of the seed bark.

Surface topography characterization and chemical analysis of the surfaces of the seeds demonstrated that while the changes in the surface topography after plasma treatment were not dramatic, the surface chemistry of the seeds changed notably as a result of the plasma treatments. Using very surface-sensitive techniques, such as photoemission spectroscopy, we showed that plasma contributes to the incorporation of a significant additional amount of oxygenated and nitrogenated functional groups, the removal of aliphatic groups and the promotion of the upward surface diffusion of potassium ions. These effects are reversed upon exposure to water, either in vapor or liquid form, a result that suggests the diffusion of these plasma-generated functionalities into the interiors of the seeds. We hypothesize that some of these diffused surface functionalities are RONS that are responsible for the observed increase in the germination capacity.

Data availability statement

All data that support the findings of this study are included within the article (and any supplementary files).

Acknowledgments

The authors wish to thank the AEI-MICINN (PID2019-110430GB-C21, PID2019-109603RA-I00), CSIC (2019AEP161, 201860E050), Junta de Andalucia (PAIDI-2020 through projects P18-RT-3480, US-1263142 and ref. AT17-6079)) and the EU through the cohesion fund and FEDER 2014–2020 programs for financial support. CLS thanks the University of Seville through the VI 'Plan Propio de Investigación y Transferencia de la US' (VI PPIT-US).

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