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RESEARCH ARTICLE

PLASMA PROCESSES AND POLYMERS

Comparative analysis of the germination of barley seeds subjected to drying, hydrogen peroxide, or oxidative air plasma treatments

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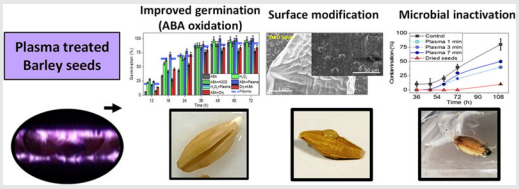
Abstract

Acceleration in germination time by 12–24 h for barley seeds treated with atmospheric air plasmas may have a significant economic impact on malting processes. In this study, the increase in germination rate and decrease in contamination level upon plasma treatment could not be directly correlated with any significant increase in the water uptake capacity, except for seeds exposed to mild drying treatment. A variety of germination essays have been carried out with seeds impregnated with an abscisic acid solution, a retarding factor of germination, treated with a peroxide solution, and/or subjected to the plasma and drying treatments. Results suggest that plasma and hydrogen peroxide treatments induce the formation of reactive oxygen and nitrogen species that affects the abscisic acid factor and accelerate the germination rate.



KEYWORDS
ABA factor, barley germination, hydrogen peroxide treatment, plasma surface oxidation

1 | INTRODUCTION

In the quest for improving agriculture culture yields, various strategies have been developed to improve germination efficiency and accelerate the first stages of plant growth. One of these strategies consists of treating the seeds with air plasmas.[1] It is already well-established that a large variety of seeds treated with air plasmas experience an acceleration in germination rate and/or an increase in the percentage of germination success.[2–12] This rather common behavior presents some exceptions where plasma treatment has been reported to be neutral or even detrimental for germination,[10,13,14] particularly under water scarcity conditions.[14] Common interpretations of these results have related the germination improvement with an increase in seed water permeation and imbibition[15–17] or with the plasma removal of contaminating biological agents.[1,17–21] Other works though relate the acceleration of germination rate with the affectation of dormancy and the effect on growth factors such as the abscisic acid (ABA) and other seed enzymes of reactive oxidative species (ROS) formed at the seed surface upon plasma interaction.[22–30] As a result, the effect of plasmas on germination is still unclear, failing a clear understanding about the incidence of plasma effects on the germination process. This situation is still more puzzling since the actual influence of plasmas on germination may also depend on the characteristics of seeds and their external coat or the treatment time and type of gases utilized to ignite the plasmas,[9,31] with case examples where plasma produced either no effect or a negative effect on water uptake[14] and/or germination efficiency.[10,13] This complexity has been further jeopardized by the difficulty to compare results and tests carried out in different laboratories and/or with various seed types of the same species.

The present work deals with barley seeds, one of the most common cereals around the world that serve as the basis for the animal and, in part, human feeding.[32,33] In addition, barley is a cereal with a high industrial impact on the production of beer and other agrofood products. Particularly for these industrial applications, any improvement in germination rate, even if meaning a small decrease in the period of time required to open the seed bark and trigger the plant rooting, may have a considerable economic impact because of an effective reduction in the malting period.[34] The enhancement of germination rate of barley seeds treated with plasmas is a widely studied case example[25,29,35–40] where, similarly to other seeds, the found improvements in germination have been attributed to a variety of chemical and physical effects of the plasma treatments either on the surface of seeds and/or in their internal biochemistry and enzymatic activity. Herein, we investigate the effect of plasmas on the germination and growth of barley seeds and study the influence on other phenomena that may be also affected by plasma and/or other related treatments. Namely, we directly investigate the changes that cold air atmospheric plasmas produce in the water uptake capacity of barley seeds both from the vapor and liquid phases. Indirectly, we also assess its effect on the ABA factor and its influence in the germination rate of seeds treated or nontreated with air plasmas. For comparison, we also study the effect of treating the seeds with an H_2O_2 solution, a classical treatment known to promote the seed germination capacity.[41–46] Similar analysis has been also carried out with seeds that have been subjected to a severe drying process at room temperature and where an increase in germination rate was also observed. This drying treatment, although not completely equivalent to classical priming treatments,[47,48] has resulted quite efficiently in accelerating the germination rate. The analysis of treated and nontreated barley seeds with techniques such as scanning electron microscopy (SEM) or X-ray photoelectron spectroscopy (XPS) has been also carried out to determine possible changes in the chemistry and/or surface morphology induced by the plasma treatments. Although these investigations do not permit yet to figure out a holistic model accounting for all phenomena resulting from the atmospheric air plasma treatments of seeds, clear evidence of this investigation is that water uptake capacity results are almost unaffected by the air plasma treatment and that the plasma affectation of the ABA present in the seeds is an important factor contributing to increase the germination rate.

2 | EXPERIMENTAL SECTION

2.1 | Seeds type

Mature barley seeds (*Hordeum vulgare* L. var. planet) provided by the company Intermalta S.A. have been used for this study. No special treatment was applied to these commercial seeds. This variety of barley seeds is typical of two-rowed and spring cycles. It is also rather common for the malting industry due to its high productivity and quality. For agricultural purposes, it is much appreciated because of its adaptability to different zones and agroclimatic conditions. During the time required for the experiments (about 6 months), seeds were kept stored in a closed container under controlled conditions of temperature and humidity.

2.2 | Plasma reactor and plasma treatment conditions

Plasma treatments were carried out in a dielectric barrier discharge reactor with a parallel plate configuration. Two stainless steel electrodes (8 cm diameter) were covered with two quartz plates (0.5 mm thickness and 10 cm diameter to avoid edge discharges). The separation between the two plates was fixed at 4.2 cm and the seeds (usually 35 seeds specimen for each treatment) were placed well separated onto the bottom grounded electrode. The active electrode at the top was activated by a high voltage source from TREK (model PD05034) that, in turn, was connected to a function generator (Stanford Research System, model DS345). V(t) and I(t) signals were recorded with an oscilloscope (TEKTRONIK, model TDS2001C) with a bandwidth of 50 MHz and a sample rate per channel of 500 Ms/s. A 1:1000 high voltage probe and a current probe coil (conversion factor of 0.05 V mA^{-1}) were used for this recording. For all treatments, the high voltage source was set at 1 kHz frequency and 8.6 kV voltage. Under these conditions, the current passing through the system was 6.5 mA and the discharge power 5.3 W. A pressure of around 700 mbar of dry air was dosed in the reactor during the discharge for periods of time fixed at 1, 3, and 7 min. Occasionally, longer times up to 15 min or more were also utilized. Further details about the reactor and operating conditions can be found in previous publications.[5,49]

2.3 | Drying procedure

Various sets of seeds were subjected to a drying process either in gentle ambient temperature (around 21°C) conditions in a desiccator or to more intensive drying treatments on a stove at 50°C and 70°C for 24 h. After these intensive drying essays, germination results were bad in the sense that only a reduced amount of heated seeds accomplished germination. Therefore, accelerated drying experiments will only be incidentally mentioned in the text. Drying at ambient conditions was carried out by placing sets of 50 seeds in a closed flask containing silica gel (Panreac), avoiding any direct contact between the silica gel granules and the seeds. Usually, after 2/3 weeks in these conditions, seeds experienced a weight decrease of about 4%–6% of its initial weight. We will designate these seeds in the text as "dry" seeds.

2.4 | Sowing tests and statistics

Germination tests were carried out under two different conditions: in plastic Petri dishes and in soil substrate. For the germination tests in the plastic Petri dishes, sets of 50 seeds (control seeds or seeds previously subjected to different treatments) were carefully placed on a double Whatman filter paper located in Petri dishes of 9 cm diameter. A watering of 4 ml per dish with miliQ water was applied to each dish. Petri dishes were then placed in the dark in a bacteriologic incubator (Selecta model PREBATEM 80L) at a fixed temperature of 20°C. After successive 12 h the Petri dishes were carefully inspected to identify seeds that might have germinated. A seed was considered "germinated" when the radicle had traversed the seed cover or upon the emergence of the coleoptile. Germinated seeds were counted and placed in another Petri dish. This process was repeated every 12 h up to completing a period of 72 h. The procedure utilized for the germination tests in Petri dish is deemed to be close to the industrial malting process of barley seeds. For comparison, germination tests were also carried out in the soil. These tests were carried out by sowing individual seeds into the alveols of horticultural cell trashes (20 seeds per test, one seed per cell at an approximate depth of 1 cm from the soil surface) filled with a commercial substrate provided by Klasmann-Deilmann GmbH 49744 Geeste, Germany (peat FLORABELLA consisting of a mixture of clay and Sphagnum blonde and black soil, this latter frozen). Physical and chemical characteristics of this soil were an electric conductivity of 40 mS m^{-1} (±25%), pH (H_2O): 5.5–8.5 and added fertilizer NPK: 14–16–18 in a proportion of 1.5 kg m^{-3}. The ensemble was placed in an illuminated climatic chamber (111 μE m^{-2} s^{-1} of photosynthetic photon flux density [PPFD] and 16 h of photoperiod) at a temperature of 24°C and constant humidity (80%). A watering of 5 ml per cell was applied three times per week (Monday, Wednesday, and Friday). By this test, seeds were considered germinated when the aerial part of the plant had just emerged from the substrate. It is noteworthy that this evaluation of germination percentage is subjected to larger uncertainties than the equivalent evaluation in Petri dish as the sowing depth in soils cannot be exactly replicated from one alveol to another. In parallel to the assessment of germination rate in the substrate, taken as the emergence time of the plant, the heights of the plants were also measured after 7 days from the planting day.

Germination in Petri dishes and germination in the substrate of seeds subjected to the different treatments carried out in this study (i.e., plasma, drying, H_2O_2, ABA, etc., see Sections 2.2 and 2.7), water uptake and contamination tests were carried out with sampling sets of 50, 20, 30, and 10 seeds, respectively. An error bar corresponding to the standard deviation over two exact replicates of each experiment has been assumed for the obtained results. When data refer to the height of plants,

we utilize the standard error of the mean value to assess the accuracy of the obtained results.

2.5 | Contamination tests

To determine the contamination degree of the seeds and its evolution with time, a set of 10 seeds was located in a laminar flux cabinet in Petri dishes of 9 cm in diameter. The utilized sterilized Petri dishes were filled with 40 ml of a mixture of agar and water (0.6%) previously sterilized in an autoclave. The seeds were examined every 24 h to verify the evolution of contamination at the seeds as identified by the presence of a fungus mycelium or a bacteria stream/colony on it.

2.6 | Water uptake tests

The capacity of seeds to incorporate water in their interior was determined following two procedures consisting of their exposure to water vapor at 100% relative humidity and to liquid water, respectively. In both cases, the uptake capacity was determined by weighing sets of 20 or 50 seeds exposed to water, vapor, or liquid respectively, for increasing periods of time.

For the water vapor uptake test, seeds were placed on a Petri dish floating onto liquid water contained in a closed flask. The temperature was kept constant at 21°C. These conditions provide a water vapor pressure of 18.7 Torr (i.e., vapor pressure of water at this temperature) and relative humidity of 100%.

For the liquid water uptake test, seeds were immersed in liquid water (milliQ quality) for given periods of time and then weighted to determine the percentage of weight increase in each case. Before measuring the weight, the seeds were left to dry on a filter paper in air for 1 min.

An additional test aiming at carefully determining the evolution with time of the uptake of water was carried out at a constant temperature of 21°C, relative humidity of about 80%, and one seed in each trial. The analysis was carried out at constant temperature (21°C) in a DSC-TGA SDT 0600 (TA Instruments) microbalance for a plasma, dry, and control seed each time. The microbalance was operated in the following way: the corresponding seed was placed in one of the arms of the device. The enclosure was then closed and nitrogen let flow through the chamber for 5 min at a flow rate of 10 ml min^{-1}. At this point ($t = 0$ for the experiments), an airflow of 150 ml min^{-1}, after bubbling through water at 21°C, was mixed with the dry nitrogen flow, and weight measurements were taken automatically and in a continuous way for the next 6 h and 15 min. Taking into account the used nitrogen and water vapor saturated air flows, this mixture renders a relative humidity of 93% for the tests. After the indicated period in humid conditions, the flow of air was closed and dry nitrogen continued flowing through the weighting chamber for more than 12 h at the same temperature. It is noteworthy that the exposure time of the seeds to the atmosphere before their placement in the thermogravimetric balance was minimized as much as possible, but was different depending on the seed. The dry seed was taken directly from the desiccator and placed in the setup that was subsequently closed (exposure time to atmosphere estimated in 3–4 min). The plasma-treated seed was taken from the plasma chamber, placed in a closed tube, and brought to the microbalance, all these processes entailing a longer total exposure time to the atmosphere of 5–6 min. During this handling period, some water adsorption from the environment cannot be discarded. The *control* seed, stored in a plastic bag without any special precaution, was placed directly into the microbalance arm.

2.7 | ABA and hydrogen peroxide tests

A series of experiments have been carried out to assess the possible role of ABA in the retardation of germination and whether plasma and drying treatments may influence this retarding enzymatic factor. Experiments consisted of following the germination of seeds previously subjected to various ABA treatments to reinforce its effect in dormancy and germination retarding, or to lessen this function by subjecting the seeds to treatments that supposedly contribute to the removal of ABA. Plasma treatments in this context consisted of the standard treatment for a period of 3 min. A brief description of these treatments and the labeling utilized for the treated seeds in each case is included next. Seeds were immediately sown after the treatments:

(a) *ABA*: Seeds were soaked in a 100 μM ABA solution for 30 min. The seeds taken from the solution were placed on a double sheet of Whatman filter paper for 30 min and then left dry in air for 20 h.
(b) *ABA-plasma*: Seeds were subjected to the treatment in (a) and then subjected to the selected plasma treatment.
(c) *H_2O_2*: Seeds were soaked in a 6% water solution of H_2O_2 for 30 min, taken off from the solution, and left dried as in (a) before sowing.
(d) *ABA-H_2O_2*: Seeds were first soaked in an ABA solution as in (a) and then introduced in an H_2O_2 solution as in (c). Seeds were sown after applying the same drying process than in (c).

(e) *H_2O*: Seeds were soaked in pure milliQ water for 30 min and then dried as in cases (a–c).
(f) *H_2O_2-plasma*: Seeds were subjected to the same treatment as in (c), then dried for 20 h and then subjected to a plasma treatment like in (b).
(g) *Dry-ABA*: Seeds were dried in the desiccator for 72 h, a period after which the seeds had lost 4.5% of their weight. Then there were subjected to the same treatment than in (a) followed by their sowing.
(h) *ABA-dry*: Seeds were subjected to the same treatment as in (a). Then they were stored in a desiccator for a period of 192 h before their sowing. The drying period in the desiccator entailed a weight decrease of 12.3% with respect to the seeds treated with the ABA solution (note that seeds will likely accumulate water during the ABA treatment).

2.8 | Assessment of the content of peroxo-like species in the seeds

A central hypothesis of this study is that peroxo-like species may affect the ABA and thus induce changes in dormancy and, consequently, germination rate.[23–30,35–45,48] It is, therefore, important to assess the content of peroxo-like species incorporated in the seeds after their treatment with a plasma of air or with a solution of H_2O_2. The peroxo-like content in the seeds was estimated by means of the classical colorimetric procedure proposed by Soares et al.[50] According to this procedure peroxo-like species formed or incorporated into the seeds can be extracted by applying the following procedure: (i) grinding 1 g of seeds in a 10 ml phosphate-buffered solution (50 mmol l^{-1}, pH=6,5) with 1 mmol l^{-1} hidroxilamine; (ii) the resulting slurry is then centrifuged at 6.000 g_n for 25 min; (iii) 3 ml of the separated liquid is then mixed with a 1 ml of titanium sulfate solution at 0.1% in a volume ratio of 20%; (iv) this mixture is centrifuged at 6.000 g_n for 25 min and the separated transparent liquid is then optically analyzed with a spectrometer. Using the Lambert-Beer law, the content of peroxo-like species was taken proportional to the absorbance at 410 nm, considering an extinction coefficient of 0.28 $\mu mol^{-1}\,cm^{-1}$ and a cuvette path length of 1 cm. Three types of samples were used to determine the content of peroxo-like species: control seeds without any treatment, seeds treated with plasma for 3 min, and seeds soaked for 30 s in a 4.5% hydrogen peroxide solution. The estimated amount of peroxo-like species determined for the H_2O_2 treated seeds was corrected by dividing the result obtained in the measurement by 1.026. This correction stems from the fact that after 30 s of immersion of the seeds in the 4.5% H_2O_2 solution, they increase their weight by 2.65% and this weight percentage should be taken into account to estimate the actual weight of the sample before soaking. For example, absorption spectra recorded for liquids extracted from seeds subjected to various treatments are reported in the Supporting Information: Figure S1.

2.9 | SEM-X-ray detector (EDX) and XPS analysis

A Hitachi S4800 SEM-FEG field emission microscope working at 2 kV has been used for the morphological analysis of seed surface and cross-section cuts, without applying any metallization protocol. Elementary and compositional maps have been studied with an EDX in an EDX-Bruker-X Flash-4010 analyzer working at 20 kV.

Chemical state characterization of the surface state of seeds, including an estimation of the atomic concentration of surface elements, has been performed by XPS analysis in a SPECS spectrometer provided with a hemispherical analyzer (DLSEGD-Phoibos-Hsa3500). Nonmonochromatic Al Kα radiation was employed to record the spectra at the normal configuration, which were obtained in the constant pass energy mode at a value of 50 eV for the general survey and 30 eV for high-resolution spectra. Calibration in binding energy (BE) has been done at the carbon functional C–H and C–C bonding groups appearing at 284.5 eV in the C1s zone.

3 | RESULTS AND DISCUSSION

3.1 | Germination tests in Whatman filters and in vitro contamination test

In Figure 1a, we show results of a germination essay in a Whatman filter that illustrate found differences in the emergency of the coleoptile of the radicle. The bar plots show the evolution of the germination rate as a function of time after the treatment with an air plasma for different periods of time and, for comparative purposes, the evolution of *control* seeds and the set of *dry* seeds, these latter are subjected to drying at ambient temperature, as described in the experimental section.

Several issues deserve comment in this figure. First, plasma treatments for 1 or 3 min are quite effective in promoting the germination of barley seeds with around a 20% and a 70% germination rate after 12 and 18 h, respectively, against 8% and 44% for the *control* seeds after the same periods of time. At 24 h, germination rates reached 76% and 68%, respectively. A small difference remains after 36 h, while for longer times only a little difference of 98% versus 94% remained between seeds plasma treated for 3 min and the *control*. Taking into

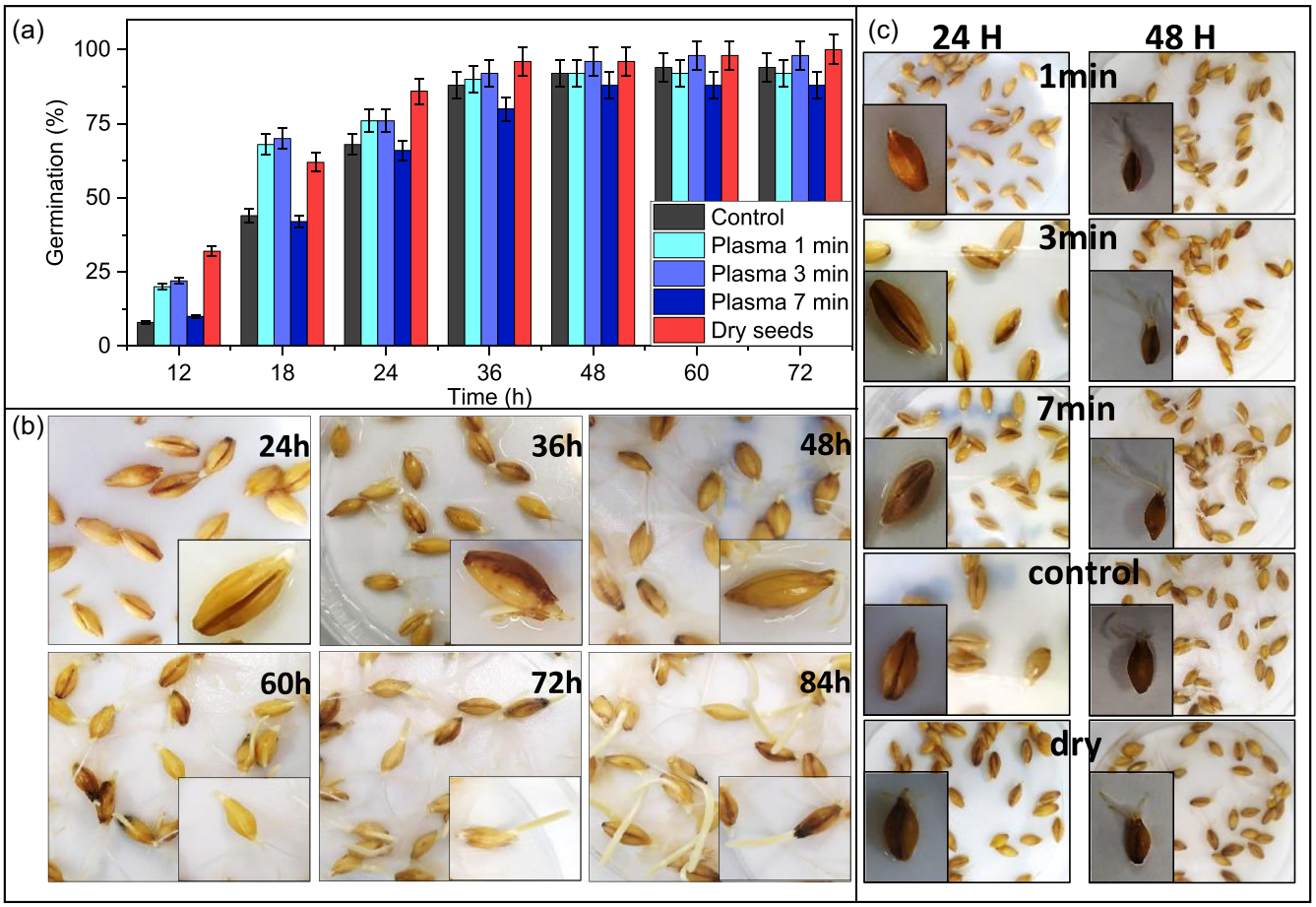


FIGURE 1 (a) Bar diagrams of the germination rate (in percentages) in a Whatman filter of barley seeds subjected to plasma treatments at various times. Results for control and dry seeds are included for comparison. (b) Images of Petri dishes for the seeds subjected to 3 min of plasma treatment for increased time periods after the treatment. (c) Images of Petri dishes for the seeds subjected to different plasma treatments at 24 and 48 h after treatments.

account the error limits considered in our analysis, it can be concluded that up to 24 h after sowing, positive differences in germination rate are clearly observed for the 1 and 3 min plasma treated seeds with respect to the *control*. It is also apparent from this plot that plasma treatments for 7 min have a certain deleterious effect in stimulating the germination rate. The images in Figure 1b illustrate the germination process at different times after sowing for seeds treated with plasma for 3 min. The progression in the emergence of the radicles is clearly seen in these images. Regarding the plasma treatment time, data in Figure 1a reveal differences within the statistical error, although a maximum 98% germination was achieved for 3 min plasma treatment. Pictures from Figure 1c corroborate the good development of the radicle as an effect of the plasma treatment compared to the control one. In the rest of this study, most experiments correspond to plasma treatment times for 3 min. Overall, the observed differences in germination rate can be of great significance when thinking in industrial manipulation where a small reduction of the malting time may have a significant impact on the cost of the process.[34]

Surprisingly, for the 24 first hours after seeding, the drying treatment produces a clear acceleration in germination rates with percentages of successful germination that were higher than those of plasma seeds. The observed differences become small after 48 h or longer times since the sowing, although plasma treated and *dry* seeds presented germination percentages between 92% and 96% against 92% for the *control* seeds. After 72 h these percentages amounted to 94%–100% for the plasma-treated and *dry* seeds against 92% for the *control*. Regarding this drying treatment, it is relevant that weighting the seeds before their storage in the desiccator and just before sowing revealed a weight loss of 4.5%, most likely due to some loss of intrinsically absorbed water. At this point, it is relevant to comment that seeds subjected to a similar desiccation process in an oven at 50°C and 70°C showed weight losses of 2.0% and 5.9%

(note that seeds were exposed to air before weighting and that they may have recovered some of the water lost during heating), but their germination was severely affected with germination rates of only 78% and 22% after 24 h (see the Supporting Information: Figure S2). As confirmed by Soares et al.[50] this behavior agrees with the known decrease in germination capacity of barley grains subjected to overheating at temperatures above 40°C. Therefore, to ensure any overheating damage, drying treatments in our work have been carried out at ambient temperature.

An important factor affecting seed germination and plant growth refer to their contamination degree,[1,18–21,51] a feature that can be positively decreased by the plasma treatments as a prevention method.[5,18,19,49] Figure 2a shows the contamination degree, defined and determined as described in the experimental section, for the plasma-treated seeds, the *control* set, and the *dry* seeds. An example of the appearance of contamination in seeds is reported in Figure 2b. Clearly, the plasma treatment produces a positive effect and contributes to drastically reducing the contamination degree with respect to the *control* seeds, with contamination percentages around 80% and 40% after 110 h for *control* and plasma treated seeds, respectively. Particularly interesting was the fact that the contamination degree of plasma-treated seeds remained under a manageable value lower than 50% even 110 h after the beginning of the test, much lower than the value found for the *control* seeds. Interestingly, the set of *dry* seeds presented an even lower contamination degree of 10% after this period of time. A hypothesis for this outstanding behavior is that the avidity of these seeds for water (see water adsorption experiments in Section 3.3) would deprive the surface of seeds of the conditions (i.e., basically hydroxylation degree and amount of adsorbed water) required for efficient deployment of fungi or bacteria. Clearly, plasma and drying treatments induce a net reduction in contamination degree with the expected benefits for seeds storage and conservation and/or their germination success.[18,19,51–53] However, whether equivalent factors are involved in reducing the contamination degree in both situations is still a subject of debate, although we presume that the oxidative character of the plasma should play a specific role in the plasma case.

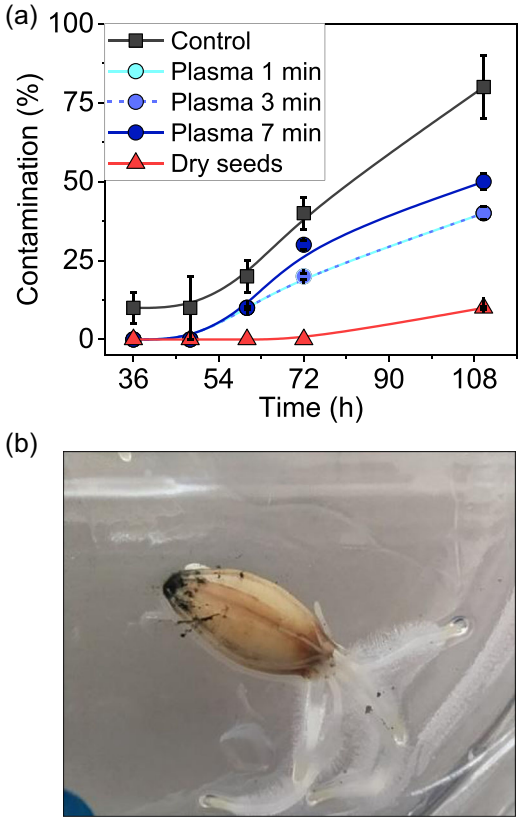


FIGURE 2 (a) Evolution with time of the contamination degree (in percentage) in vitro of barley seeds subjected to plasma treatments for various times. Results for control and dry seeds are included for comparison. Results of plasma 1 min and plasma 3 min experiments present similar values. (b) Image of a contaminated seed depicting the typical black stains that denote contamination.

3.2 | Germination rate in the substrate

The positive effect of the plasma treatment on the germination rate was less apparent for the germination essays in the substrate. It has been noted in the experimental section that small differences in the sowing depth for each seed and the fact that plants have to emerge from the soil to determine a germination event makes this analysis intrinsically less accurate than that in a Petri dish. Nonetheless, a certain favorable effect in increasing germination rate can still be noted for plasma-treated seed at 72 and 96 h after sowing (note that the criterion defining germination, in this case, is different than in the Whatman filter test and that no information is available for periods of time shorter than 72 h). According to Figure 3a, seeds treated with an air plasma for 1 and 3 min depicted a germination rate of 80% and 75% after 72 h after sowing, against 65% for the *control* and 7 min plasma treated seeds and only 30% for the *dry* seeds. Although the reasons for this slow growth rate of dry seeds are still unclear, we speculate that plant emergency is slower for these seeds (note that plant emergency is the criterion adopted to count germinated seeds in soil), while radicle emergency might occur more readily. Differences between plasma treated and *control* seeds became smaller after 96 and 120 h, although the germination rate was still slightly higher for the former. An evaluation of the height of the plants after 7 days from sowing revealed a slight favorable tendency for the

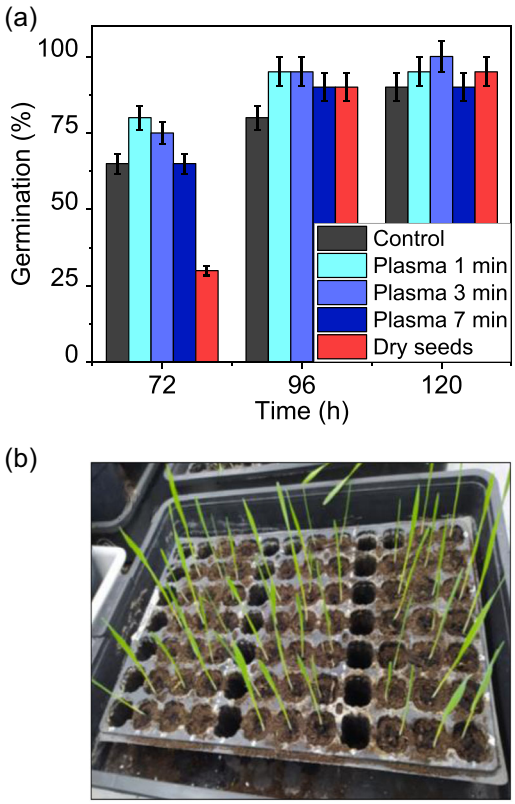


FIGURE 3 (a) Bar diagram of the germination rate in the substrate (in percentages) of barley seeds subjected to a plasma treatment at various times. Results for control and dry seeds are included for comparison. (b) Photographs of plant emergency in the trash with most plants emerged corresponding to barley seeds treated with plasma for 1, 3, and 7 min.

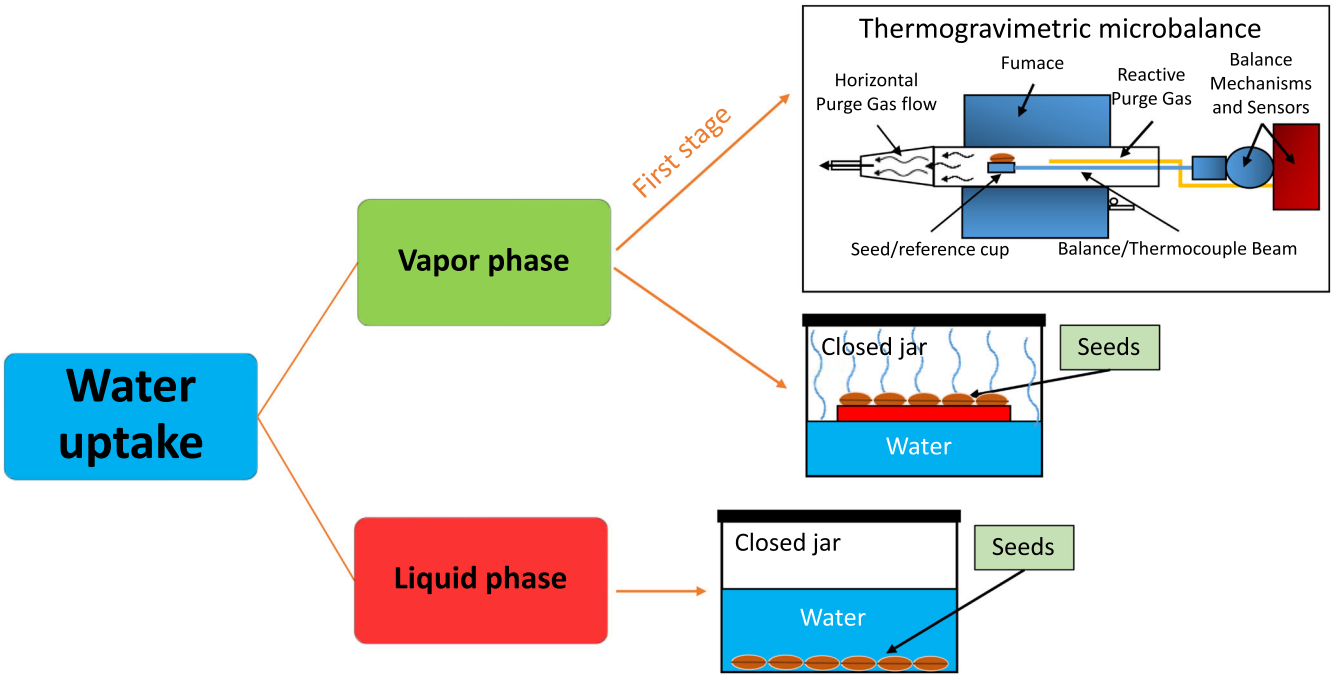
plasma-treated seeds with respect to control (see Figure 3b and Supporting Information: Figure S3) and a significant decrease in height for the *dry* seeds, in line with the observation of slower germination in soil. This latter result shed doubts about the interest of the drying treatment for the plant growing in soil, although it may still represent a clear advantage for industrial malting processes where the germination protocol presents clear similarities with the Whatman filter germination procedure.[34] For the treatments and experiments carried out in the rest of this study, as well as for the discussion of results, the reference procedure will be that of the Petri dish experiments.

3.3 | Water uptake capacity

Unlike the hydrophobic character of pristine seeds, seeds subjected to plasma treatment depicted a hydrophilic behavior (see the Supporting Information: Figure S4) that lasted several weeks after the plasma exposure. This behavior is commonly encountered for different kinds of seeds and experimental conditions and has contributed to the rather widespread idea that the water uptake capacity of plasma-treated seeds becomes exalted.[14,16,17] However, we believe that this behavior is by no means common and very likely depends on seed treatment and manipulation conditions. Actually, in previous works of our group and others working with soybean, wheat, quinoa, nasturtium, or cotton seeds it has not been found a clear increase in water uptake capacity after plasma treatments similar to those applied in the present work, while differences were quite dependent on manipulation conditions or water availability in the environment.[7,14,50,54]

For the barley seeds investigated in the present study, we have carried out a systematic investigation of their water uptake capacity either from a vapor or liquid phases, as represented in Scheme 1. The number of used seeds depended on the water uptake test: from one in the microbalance to 20 and 50 seeds in water vapor exposure and liquid water immersion experiments, respectively. The initial steps of water incorporation from the vapor phase have been also investigated in a precision thermogravimetric balance following the methodology explained in the experimental section. Recent and past publications have systematically dealt with the problem of water uptake either from liquid and vapor phases due to its importance for germination, seed storage, or long-term preservation.[55–57] Our purpose herein is more limited and aims at establishing possible differences in the water uptake capacity depending on seed treatment.

Figure 4 shows the evolution of water uptake from the vapor phase for barley seeds subjected to plasma treatment, the *control* group, and the set of *dry* seeds subjected to the drying process. The curves, obtained by weighting according to the protocol explained in the experimental section show progressive incorporation of water from the vapor phase in a continuous process extending for more than 144 h (8640 min). This progressive incorporation of water is rather similar for the *control* and plasma-treated seeds, though, depending on treatment time, plasma-treated seeds seem to uptake 2%–3% more water than the *control* seeds after similar periods of time. In turn, the *dry* seeds were much more effective to incorporate water in a process that is quite noticeable already after 24 h of exposure (weight increase by a 4.25% for a 2.7% for the *control*), presenting a clear difference after 144 h of exposure to water vapor when a weight increase of 35% was found for the *dry* seeds against 27.5% for the *control*. It is noteworthy that the germination process may also occur by exposure to humidity under the same conditions as in this experiment (see Supporting Information: Figure S5), although the germination time was longer than 168 h and



SCHEME 1 Water uptake experimental configurations, from vapor and liquid phases

presented a well-differentiated behavior depending on seed treatment.

The incorporation of water from the vapor phase was also investigated in a model experiment using a highly sensitive thermogravimetric balance operated as explained in the experimental section. The curves in Figure 5 show a comparison of the evolution of the relative weight of a *control* seed, a seed subjected to plasma treatment, and a *dry* seed (i.e., stored in the desiccator for 30 days). These curves show the relative weight evolution (i.e., $[W + \Delta W]/W$, where W represents the weight of the seed before the test) of each seed exposed first to humid air and then to the dried nitrogen flow as explained in the experimental section. We attribute the weight increase/decrease curves in this figure to water uptake/release processes. The three curves depict quite different profiles. In agreement with the evolution in Figure 5, the enhancement in the relative weight of the seed is maximum for the dry seed (i.e., 3.5%) followed, at a much lower level, for the control (0.9%) and finally for the plasma-treated seed (0.3%). Conversely, the weight loss of the seeds when closing the humid air flow, once reached the maximum weight, shows that water is more effectively retained in the dry seed (weight loss by 1.2% after 1000 min) and more easily released in the *control* (weight loss by 1.4%) and, particularly, in the plasma-treated seed (1.9%). These curves sustain that the *dry* seed presents a high avidity for water, much higher than that of the control and plasma-treated seed. In contrast, water release was maximum from the plasma-treated seed where it seems to occur readily in dry ambient conditions. We speculate that the new oxygen functional groups generated onto the surface and their hydrophilic behavior induce by the plasma treatment seem to favor water permeability, particularly under dry environmental conditions. However, at this stage, it is not justified to make a quantitative analysis of these curves because there is no data regarding the water uptake/release of seeds during their handling in the atmosphere before their placement in the set-up, time inside the plasma chamber or simply the relative humidity of the air during manipulation outside its storage container. Despite this, a qualitative evaluation of the found results is illustrated in the cartoon in Figure 5b showing this different behavior: the *dry* seed is more prone than the other to uptake water, while in the plasma-treated seed water released in dry conditions is maximum. Concerning the plasma treatment experiments, these results prove the high sensitivity of plasma-treated seeds to vapor pressure and the need to keep strict control of the ambient humidity when handling seeds in air to reach reliable results.

The water uptake capacity of seeds was higher and occurred more rapidly when they were immersed in liquid water. Figure 6 shows the evolution of weight for the seeds immersed in water for increasing periods of time. The comparison between the plasma-treated, *control*, and *dry* seeds reveal a rather similar water uptake capacity for the *control* and plasma treated seeds and a higher capacity for the dried seeds, manifested

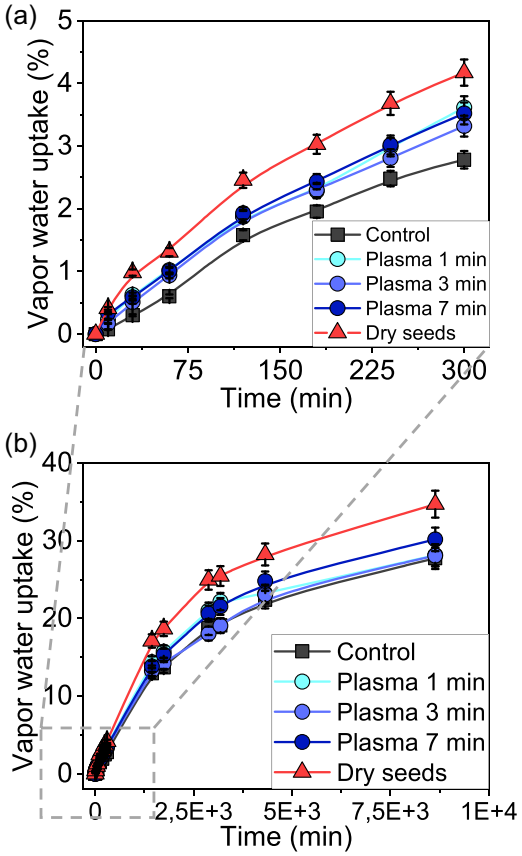


FIGURE 4 Evolution of weight in percentages of the indicated sets of seeds exposed to water vapor at 21°C for the indicated periods of time: (a) representation from 0 to the first 300 min of exposure, (b) representation from 0 to 8000 min of exposure. The dry seeds had lost 4.5% of the original weight during the drying treatment.

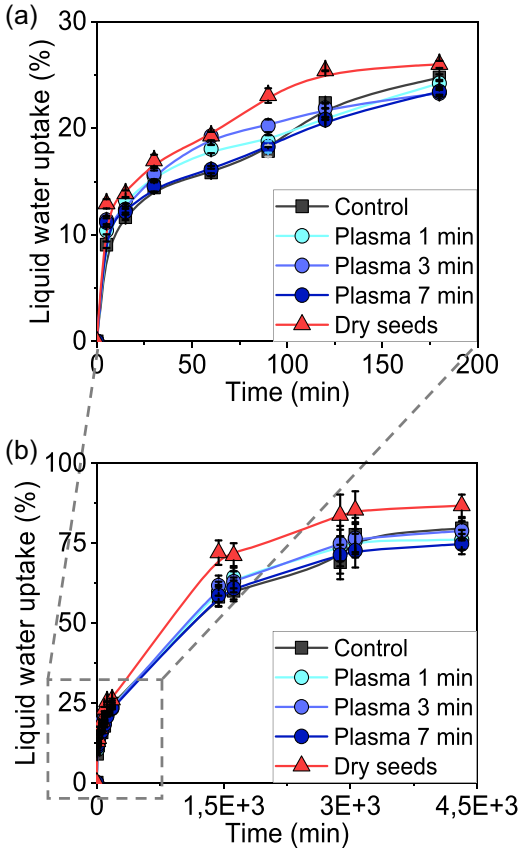


FIGURE 6 Evolution of weight in percent of the indicated sets of seeds exposed to liquid water at 21°C for the indicated periods of time. (a) Representation from 0 to 200 min of immersion. (b) Representation from 0 to 4500 min of immersion. The dry seeds had lost (4.5 ± 0.5) percent of the original weight during the drying treatment.

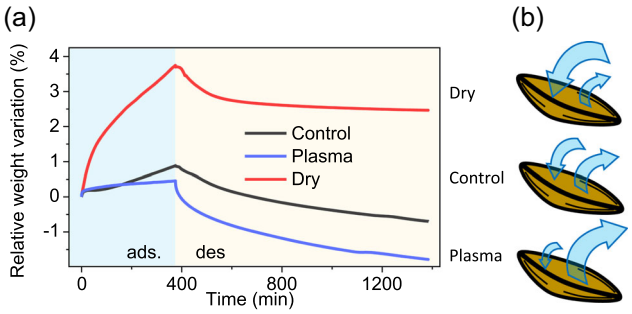


FIGURE 5 (a) Relative weight variation measured for dry, control, and plasma treated seeds in a precision balance at 21°C upon exposure to water vapor (blue zone) and to dry nitrogen after reaching the maximum weight. (b) Qualitative interpretation: Dry seeds have a tendency to incorporate water in humid conditions; Plasma treated seeds lose water easily when placed in a dried environment.

both for short (Figure 6a) and long (Figure 6b) immersion times. Longer essays were not carried out because after 72 h (around 4320 min) seeds start to germinate.

Most remarkable from these water uptake experiments is that no large differences are found for the plasma-treated and the control seeds, while a significantly higher water uptake capacity is found for the seeds that had been stored in a desiccator at ambient temperature (*dry* seeds). Interestingly, the found increase in weight for these seeds was higher (86.7 ± 3.5%) than the loss experienced by them during their drying process. This result suggests that drying at ambient temperature must trigger a compensating mechanism favoring the water uptake and, likely, the germination capacity of seeds in contact with liquid water.

3.4 | Surface morphology and chemical state of plasma treated seeds

Although the plasma utilized for the different experiments was relatively mild, different hints suggest a certain affectation of the surface of barley seeds by the utilized plasma discharge. Figure 7 shows a series of SEM micrographs taken for a *control* seed and a seed subjected to plasma treatment for 1, 5, and 15 min. These images do not show quite dramatic changes in surface topography, although in the plasma-treated seeds the progressive removal of some nodules is clearly distinguishable at the surface. A general roughening and surface damage is also observed in the seed subjected to the longest plasma treatment. The zones occupied by these nodules seem to be enriched in silicon as revealed by the EDX analysis of these seeds (see the Supporting Information: Figure S6). Overall, the SEM analysis sustains a certain affectation of the surface of barley seeds by the plasma treatments, in line with the previous evidence reported in similar studies.[29,58] It is likely that this affectation of the surface of seeds exposed to plasma makes easier the out-diffusion of water from the interior of seeds to the exterior, as evidenced by the experiment in Figure 5 when no water vapor was present in the environment.

In addition to this analysis of surface state by SEM, cross sections of seeds were also examined by SEM and EDX (see the Supporting Information: Figure S7). The obtained results revealed that a certain enrichment in minority elements such as K and P was induced by the plasma treatment at the external zones of bark. Similar behavior has been found by us for quinoa and cotton seeds exposed to plasma treatments.[5,49] These evidence revealed that although the surface topographic changes induced by plasma are not quite significant, a certain mobilization and out-diffusion of some elements takes place as a result of this treatment. We tentatively propose that the observed diffusion of elements likely responds to changes in chemical potential induced at the surface by the plasma treatments.

The previous results about modification of the profile distribution of minority elements upon plasma treatments complement the inferences on chemical changes observed by XPS at the outermost surface of seeds (this technique proves about 1–2 nm of thickness). Figure 8 shows selected spectra taken at the O1s, C1s, and N1s peaks for the *control* and *dry* seeds, and for the seeds subjected to plasma treatment for 3 min. Other elements identified at the surface of seeds were Ca and Si, whose spectra are reported in the Supporting Information: Figure S8. A quantitative estimate derived from these spectra of the percentage of the different elements at the surface is reported in Table 1. The XPS analysis revealed that plasma treatment modified slightly the chemical state of the surface as evidenced by a small increase in the concentration of COx surface groups (contribution around 288 eV highlighted with an arrow in the figure[5,20,59]) and the increase in the relative concentration of oxygen at the surface (see Table 1). Besides this surface oxidation, the analysis of the N 1 s signal also demonstrates the incorporation on the surface of new nitrogen functionalities as deduced from the increase in

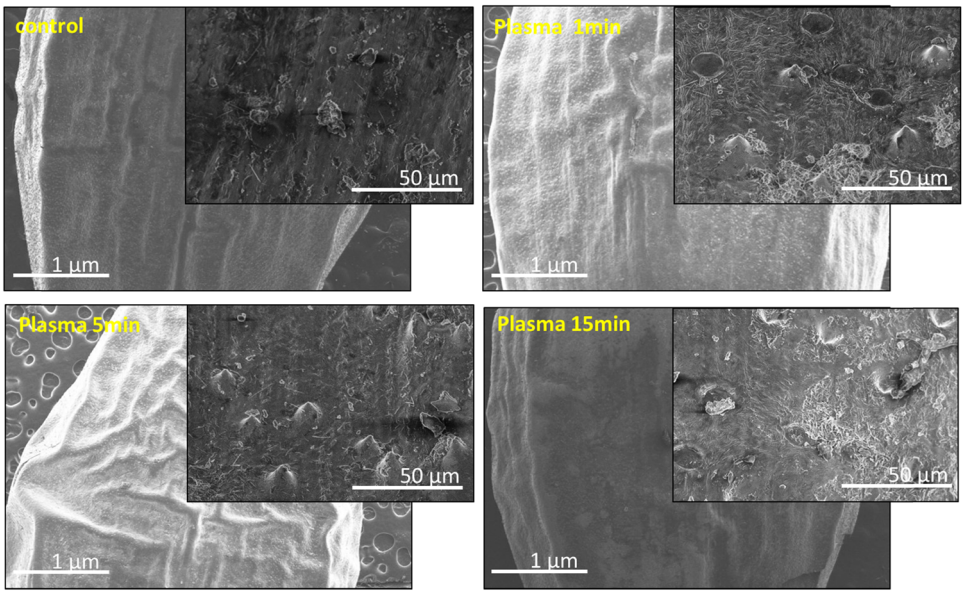


FIGURE 7 Scanning electron microscopy micrographs at two different magnifications (see the scale bar in each case) for a barley seed in its original untreated state, and after treatment with plasma for the indicated periods of time (1, 5, and 15 min).

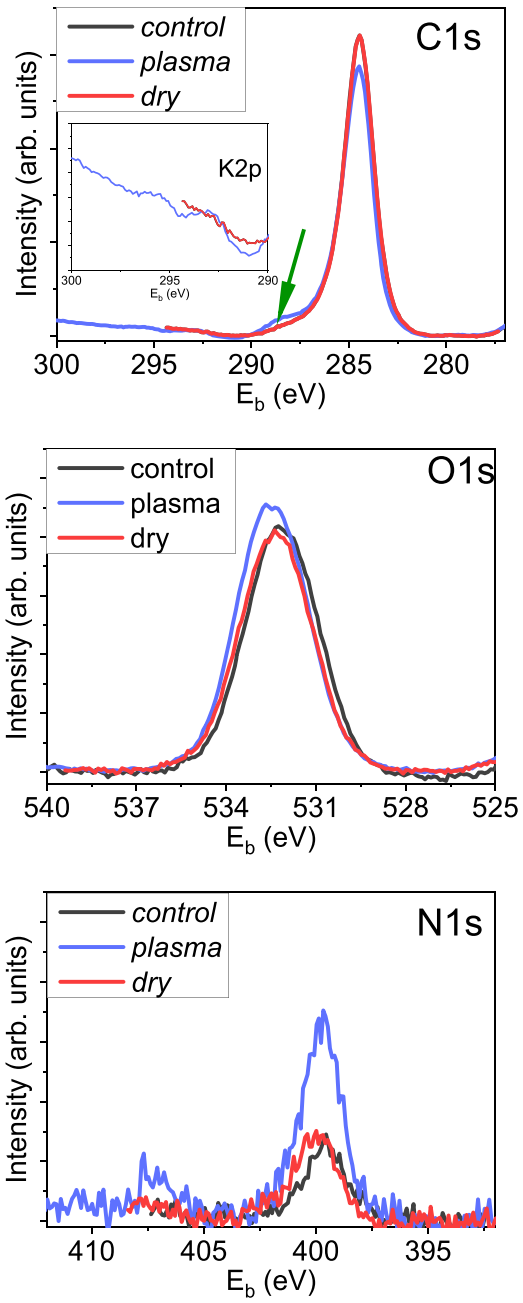


FIGURE 8 Photoelectron spectra of the C1s, N1s, and O1s levels for the control, plasma-treated, and dry barley seeds. The inset in the C1s panel shows an enlargement to highlight the evolution of *K2p* at the surface after the plasma treatment. The arrow in this panel shows the evolution of a shoulder in the plasma-treated seed.

the intensity of the peak at around 400 eV attributed to C–N bonds and the appearance of a new band at around 407 eV due to NOx species.[5,20,59] Interestingly, the spectrum around the C1s zone of the plasma-treated seeds also shows the evolution of the characteristic K 2p signal, indicating a certain outwards surface diffusion of this cation when the seeds are treated with plasma.

TABLE 1 Atomic percentages of the different elements detected by X-ray photoelectron spectroscopy at the surface of barley seeds after the indicated treatments

	C	O	N	Ca	Si
Control	87.5	10.0	0.7	1.1	1.2
Plasma 3 min	79.1	16.5	1.5	0.5	2.4
Dry	87.5	10.1	0,7	0.3	1.4

Qualitatively, similar results have been also found for other seeds investigated in our laboratory in what seems to be a general behavior of seeds subjected to air plasma treatments.[5,49] In addition to these changes, XPS analysis of barley seeds showed that plasma treatment produced a certain increase in the surface concentration of silicon and evidenced the presence of small and variable quantities of calcium at the surface depending on seed treatments (see Table 1). The relative increase of silicon agrees with the partial loss of the surface nodules, which might be covering zones rich in Si and become detached after the plasma treatments, as revealed by the SEM-EDX analysis (see Supporting Information: Figure S6).

For the purposes of the present study, the most interesting is that plasma treatment entails the partial oxidation of the seed surface, indicating that the active species of the plasma are interacting with the carbonaceous material of the bark surface. This partial oxidation and the formation of COx functional groups at the surface are likely responsible for the transformation of the seed surface into hydrophilic (another factor might be the partial removal of wax during this treatment). However, according to the results in Figures 4–6, this transformation of the surface state of seeds does not seem to significantly increase their water uptake capacity. It is also noteworthy that neither particular surface oxidation nor a change in the silicon concentration at the surface could be detected by XPS for the *dry* seeds (data not shown).

3.5 | Germination factors and the effect of atmospheric air plasmas

The content of the ABA phytohormone and other enzymes in the seeds is generally recognized as a dormancy factor controlling the germination and growth processes.[23–30] Their presence in the seeds has been taken as directly linked with the germination rate. Therefore, in this study, we have tried to determine the possible effect of plasmas on the deactivation/removal of ABA and, consequently, with the observed increase in

the seed germination potential. In general, a factor claimed to control the germination rate of seeds subjected to various activation treatments including plasmas is the affectation of the ABA germination factor by ROS and/or reactive nitrogen species (RNS), or a combination of both (RONS) generated by these treatments. This can be directly inferenced when treating the seeds with H_2O_2,[41–45,48] but it is not so evident for the plasma treatments where the role of RONS in activating the germination is still a likely hypothesis.[5,49] In the present study with barley seeds, hints by XPS suggest the formation of similar species upon plasma treatment. In recent work, the formation of very reactive radical species in barley seeds subjected to plasma treatments has been proved by electron paramagnetic resonance.[35]

To prove a given affectation of ABA by the plasma treatment through its reaction with the RONS formed by plasma treatment (from now we will utilize the term RONS in a generic way, referring not only to stable ROS and RNS species but also to radical species with high chemical activity as expected for a plasma environment), we applied an alternative methodology to check whether these reactive species may diffuse and react with the ABA and contribute to accelerating the germination process. A series of germination experiments were carried out with the various set of seeds, as described in the experimental section: *ABA, ABA-plasma, H_2O_2, ABA-H_2O_2, H_2O, H_2O_2-plasma, Dry-ABA, and ABA-dry*.

Results of the germination rate essays carried out with these differently treated seeds are reported in Figure 9. According to the bar diagram in this figure, germination starts after 12 h, but with a quite different success depending on seed treatment. In general, ABA treated seeds presented a much lower germination rate than the *control* seeds (c.f. Figure 1) and, particularly, than the set of seeds treated with H_2O_2, plasma, or successively both of them. Interestingly, this occurs even for seeds that had been previously treated with the ABA solution. In other words, while the ABA treatment contributes to reducing the germination rate, the plasma and H_2O_2 treatments contribute to enhancing germination and are able to compensate, and even neglect the effect of ABA. The observed evolution of germination rates suggests the following: (i) in addition to its natural content, some amount of ABA may become incorporated into the seeds after their impregnation with this germination retarding factor, producing an additional retarding effect on the germination rate (see results for *ABA* and *Dry + ABA samples*); (ii) the treatment with plasma or H_2O_2 compensates this effect, likely producing the removal and/or inactivation of the ABA factor (see results for *ABA + plasma* and *ABA + H_2O_2 seeds*); (iii) the direct treatment of the seeds with either plasma + H_2O_2 or H_2O_2 produces an exaltation of the germination rate, likely because these treatments contribute to remove and/or inactivate the intrinsic amount of ABA present in the seeds. All this evidence suggests that both the plasma and the H_2O_2 treatments partially affect or degrade the ABA producing a similar effect on the germination rate of seeds.

3.6 | Plasma treatments and improvement of germination rates

Treatment of seeds with H_2O_2 is recognized as an effective procedure to accelerate the germination rate,[41–45,48] although for high H_2O_2 concentrations a deleterious germination effect is also observed.[42,43] The positive effect of H_2O_2 in increasing the germination rate is usually attributed to the removal oxidation of the intrinsic ABA factor present in the seeds due to the effect of peroxide and/or hydrogen peroxide species that, adsorbed on their surface, may diffuse to the interior and act as effective ROS, degrade the ABA factor and therefore contribute to trigger and accelerate germination. The set of experiments in Figure 9 suggests that plasma acts similarly to the H_2O_2 treatment and, in this way, supports the fact that the main reason for the positive effect of plasma in accelerating the germination

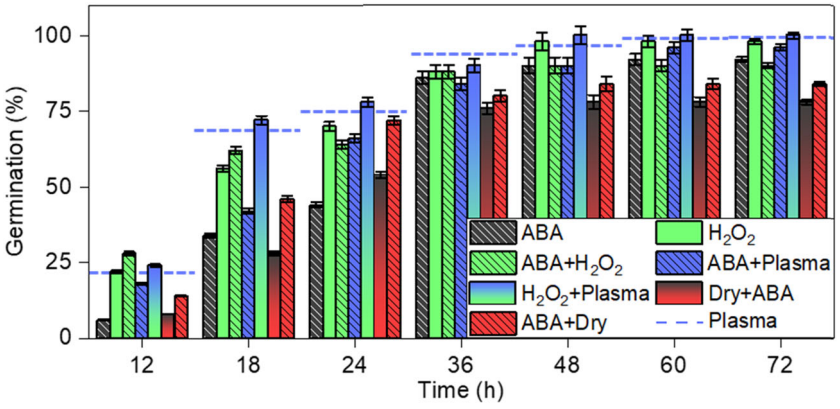


FIGURE 9 Germination rate evolution as a function of time for the indicated seeds. Bar plots are shown for the intervals comprised between 12 and 72 h after sowing. The dashed horizontal line for each time corresponds to the germination rate of plasma-treated seeds for 3 min.

of seeds is the formation of RONS species on the surface of seeds and their diffusion and reaction with ABA, as previously suggested in other works using plasmas.[5,49] However, to our knowledge, no other works in literature have carried out a similar empirical study comparing the effect of H_2O_2 and plasma in relation to the inactivation of ABA, as evidenced by the data in Figure 9.

A direct assessment of the formation of peroxide and similar species on the surface of seeds is possible by the well-known Jana and Choudhuri method.[52] Although this essay does not discard the detection of other seed components released simultaneously with the peroxo-like species,[20] it provides a semi-quantitative estimate of the amount of these latter in the seeds. Determination of peroxo-like and other species generated by plasma or H_2O_2 treatments has been also carried out in recent works on other seeds.[60,61] The application of this test to seeds exposed to a plasma, treated with the H_2O_2 solution or just in their pristine state gave the results reported in Table 2, where the rather similar values were found for the seeds treated with H_2O_2 or with the plasma are higher than the value determined for the *control* seeds. This analysis clearly indicates that in both plasma and H_2O_2 treated seeds the concentration of peroxo-like species is higher than in the *control*, thus supporting the assumption that this extra concentration of peroxo-like species has inactivated some ABA factors and promoted the germination rate of these seeds (c.f., Figure 9).

Based on these evidence, we propose the scheme in Figure 10 to account for the mechanism involved in the promotion of germination in plasma-treated seeds. According to this scheme, H_2O_2 (upper line) and RONS (middle line) plasma species contribute to the removal of a certain amount of ABA from the seeds that, in this way, experience an acceleration in their germination rate in comparison with the seeds treated with ABA (bottom line). It should be mentioned that according to the superficial character of the plasma treatment, the effect on ABA is expected to be predominant in the outermost region of the seed surface and diffusing toward the interior.

3.7 | Drying of seeds and improvement of germination rates

Another important evidence in the present study has been the positive effect in the germination of the drying treatment at ambient temperature and its effective role as a priming treatment for the germination tests carried out in Whatman filters. According to the data in Figures 4–6, the drying process at ambient temperature produces an enhancement of the water uptake capacity of seeds to an extension that overpassed the water removal induced by the drying treatment. Since no plasma formation of RONS occurs upon this drying treatment (although the formation of other kinds of similar oxidative species cannot be discarded as resulting from the stress conditions of the drying treatment,[62]) the beneficial effect of the drying treatment must be primarily associated with the rapid uptake of water (i.e., adsorption and diffusion) found in this case, rather than with any particular affectation of the intrinsic ABA content in the seeds. According to Angelovici et al.,[63] many of the enriched biological processes occurring during seed desiccation may also contribute to germination. This hypothesis is supported by the reported evidence that radicle protrusion during germination does not require the synthesis of mRNAs, which are likely present in the stored mRNA population of dry seeds. Specifically for barley seed, it has been shown by Sharanagat et al.,[57] that there is a smooth transition of transcription programs between late seed maturation and germination within the embryo, but not in the endosperm and/or aleurone, indicating that the initiation of some germination programs begins already in the desiccating embryos. In fact, many genes involved in these processes are downregulated during seed desiccation representing apparently the termination of seed maturation. Therefore, we hypothesize that the attenuation of, at least, some of these metabolic processes by the rapid seed rehydration, may trigger seed germination. It is noteworthy in this regard, that drying treatments carried out at elevated temperatures were rather deleterious regarding the germination rate, despite that the weight loss was rather similar in all cases. These experiments suggest that heating at high temperatures, though producing a certain decrease in water content, may also affect the water diffusivity through the seed membrane and protein content as reported by Soares et al.,[50] resulting deleterious for the germination process. Quite interesting in this regard is that the *ABA + dry* seeds (Figure 9) presented a smaller germination rate than the *dry* seeds (i.e., not subjected to any previous treatment with ABA), clearly suggesting that the incorporation of an extra amount of ABA retards the germination and neglects any positive effect of the water uptake in the acceleration of

TABLE 2 Concentration of peroxo-like species in the seeds subjected to the plasma and H_2O_2 treatments

	Control seeds	Plasma treated seeds	H_2O_2 treated seeds
Peroxo-like species μM H_2O_2	1.15 ± 0.08	1.71 ± 0.01	1.945 ± 0.173* (1.90 ± 0.18)

*Value after correction as explained in the experimental section.

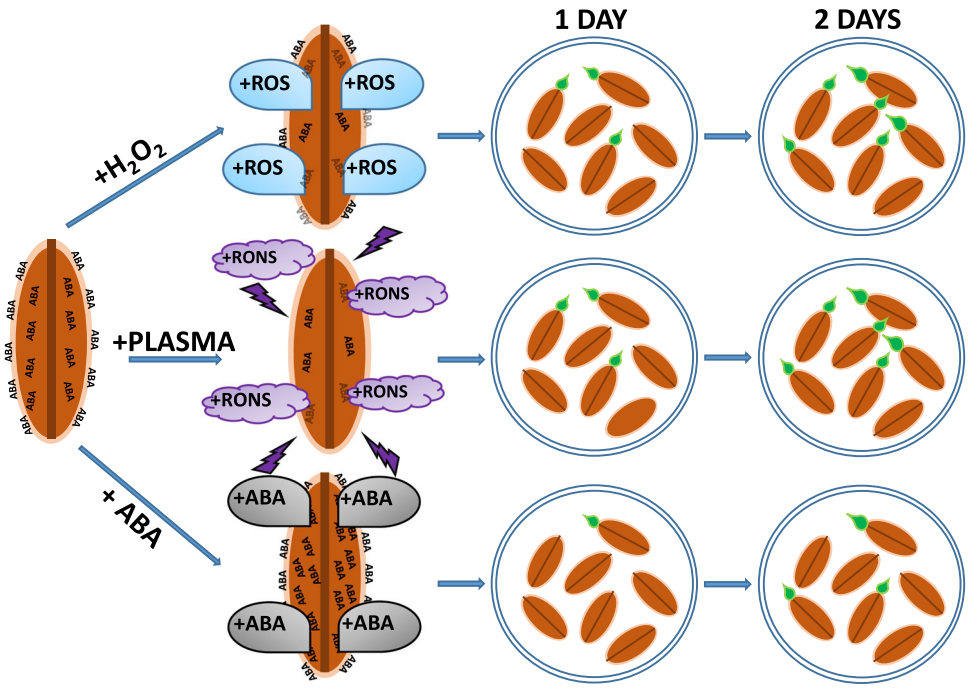


FIGURE 10 Scheme showing the effect of H_2O_2 and plasma treatments in the generation of reactive oxygen and nitrogen species (RONS) and the affectation of abscisic acid (ABA) as responsible for the acceleration of the germination rate. From left to right: the removal of ABA molecules after the incorporation of RONS species produces a decrease in the concentration of ABA within the seeds and the triggering of germination evidenced by an increase in the germination rate.

germination rate. However, the reported evidence in this study depicts a rather complex landscape that calls for additional analysis of water uptake processes in seeds subjected to various drying treatments, the characterization of the membrane behavior of the seed bark, and related experiments that should be the subject of future works.

Regarding the effect of plasmas, we should also mention that the incorporation of water into the seeds before their plasma treatment can be also deleterious for germination. In the Supporting Information: Figure S9, we show that the germination rate of barley seeds, which were water impregnated and then subjected to the same plasma treatment as the pristine seeds, experiences a significant decrease in germination rate. Although several factors might account for this behavior, we tend to attribute it to the formation of high-intensity discharges focalized onto the seeds that, recognized by the appearance of dark spots on their surface, suggest the affectation of their germination capacity.

Overall, the evidence and empirical findings in the current study define a complex portrait of the factors involved in the acceleration of seed germination capacity upon plasma treatment. For example, no similar effects should be expected whether the plasma treatment is carried out at atmospheric pressure, where neither a long waiting period before the treatment nor a significant drying effect is expected, or at low pressure as in this latter case an efficient removal of water to achieve the vacuum required to ignite the plasma might act as a drying treatment in a similar way than the drying process carried out here. This might imply that the benefits of this low-pressure plasma treatment would be due to a "drying" effect or to the combination of the drying plus the plasma, rather than to the sole effect of RONS species formed by plasmas.

4 | CONCLUSION

The previous results and discussion have shown that the germination rate of barley seeds can be accelerated by a mild air plasma treatment at atmospheric pressure. Alternatively, a similar acceleration of seeds germination rate has been found for barley seeds subjected to a drying treatment in a desiccator at ambient temperature. Variations in contamination degree have been also found for the differently treated seeds in such a way that those exposed to plasma or subjected to the drying treatment were less affected by fungi contamination than control seeds. Beyond describing these behaviors on an empirical basis, most experiments carried out in this study have tried to prove the influence of various factors in accelerating the germination rate. Unlike the rather common interpretation of the effect of plasmas in accelerating the germination rate as resulting from an increase in the water uptake capacity, our water uptake

results from liquid or vapor sources indicate that water incorporation is quite similar in the plasma-treated seeds and in the control, although it significantly increases in the seeds dried at ambient temperature. These results suggest a possible affectation of the germination rate because of a plasma-induced modification of the water incorporation capacity, but also that this factor might contribute to the observed increase in germination in the plasma-treated seeds. Surface analysis of seeds subjected to plasma treatments indicates small changes in chemistry and topology that comply with partial surface oxidation of seeds and suggest the incorporation of RONS due to seed-plasma interactions. Based on the hypothesis that these oxidative species may affect the ABA germination retarding factor present in all the seeds, a series of treatments of seeds with ABA solutions, hydrogen peroxide solutions, and plasma and drying processes, have clearly confirmed the retarding role of ABA regarding germination and that this effect is released if the seeds are subjected to an oxidative treatment, either with H_2O_2 or air plasma. The obtained results have effectively confirmed that oxidative plasmas behave in a rather similar way to hydrogen peroxide regarding both the triggering of germination and the incorporation of oxidative species into the seeds. In this way, a general conclusion of this study is that atmospheric pressure plasmas accelerate the germination rate of barley seeds because they affect the biochemistry of seeds, very likely contributing to reducing the concentration of ABA germination retarding factor.

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DATA AVAILABILITY STATEMENT

Data are available on request from the authors.

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