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Plasma-based treatments regulate seed germination in *Cannabis sativa* via oxidative status modulation and transcriptional changes

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ABSTRACT

Cannabis sativa (hemp) is an industrial crop with expanding applications in the agrifood, textile, and pharmaceutical sectors. Despite its economic potential, seed germination and quality, essential for plant development and crop establishment, are suboptimal. In this context, plasma-induced seed priming represents a novel approach to improve germination efficiency. Although these treatments are rapid, their efficacy depends on the careful optimization of several operational parameters. Here, we aimed at developing optimized plasma priming treatments for hemp seeds by evaluating the influence of gas composition, operating pressure, and treatment duration. Two seed lots of a commercial hemp variety with distinct seed quality levels were used. The physiological effects on seed germination were determined by measuring several parameters (germination percentage, rate, and speed) while additional analyses included the levels of water uptake, surface hydrophilicity, chemical modifications, oxidative status, and selected gene expression profiles. Integrative data analysis revealed that oxygen-based plasma applied at high power intensity and short exposure time resulted in enhanced germination performance, likely due to increased hydrophilicity and subsequent water uptake. This treatment likely acted as a priming agent, stimulating pre-germinative metabolism, as evidenced by the transcriptional profiles of target genes. However, prolonged exposure resulted in detrimental effects possibly attributed to an excessive water uptake and ROS over-production. Overall, these findings suggest that properly calibrated plasma treatments can stimulate beneficial physiological responses and enhance germination, whereas excessive exposure disrupts cellular homeostasis and compromises seed performance.

1. Introduction

Cannabis sativa (hemp), a dioecious plant of the Cannabaceae family, is recognized as one of the earliest species to be domesticated and cultivated (Long et al., 2017; McPartland, 2020; Ren et al., 2021). Its sexual dimorphism, lengthy domestication, and adaptation to diverse climatic conditions have led to the development of numerous varieties with distinct phenotypic traits and applications in the textile, biomedical, cosmeceutical, and agri-food sectors (Piluzza et al., 2013; Ren et al., 2021). The primary products of the hemp value chain are its fibers and seeds. Natural hemp fibers are experiencing a resurgence in market demand due to their sustainability, cost-effectiveness, high tensile strength, and stiffness (Manaia et al., 2019). Hemp seeds are a rich source of macronutrients such as carbohydrates, proteins, and lipids, making them nutritional food and feed resources (Alonso-Esteban et al., 2022; Burton et al., 2022). However, hemp cultivation remains constrained by limited knowledge regarding physiological development, particularly during the critical germination phase (Langa et al., 2024).

Germination includes the activation of seed metabolism and cell proliferation, concluding with the visible protrusion of the radicle through the seed coat. A successful and uniform germination can directly determine crop establishment and influence the final yield (Finch-Savage and Bassel, 2016). Given these premises, developing methods to improve seed germination can substantially optimize agronomic practices and ultimately increase productivity. This is the case with seed priming, a pre-sowing treatment designed to enhance

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germination performance by preconditioning the seed pre-germinative metabolism (Pagano et al., 2023). By impacting early metabolic reactions, seed priming accelerates and synchronizes germination, leading to more rapid and uniform seedling emergence (Cañizares et al., 2025). Seed priming methods can be classified in chemical, physical, and biological approaches. While chemical priming involves treating seeds with solutions that modulate water uptake or provide protective compounds (e.g., osmotic agents, phytohormones, nutrients), biological priming is based on the use of beneficial microorganisms, such as plant growth-promoting bacteria or fungi (Singh et al., 2023; MacDonald and Mohan, 2025). When some of these priming methods (e.g., hormo-priming with giberellic acid, chemopriming with chlorine dioxide or potassium nitrate) were applied to *Cannabis* seeds, their rate of success has been quite variable (Islam et al., 2022; Langa et al., 2024). Physical methods typically involve exposing dry seeds to specific energy fields, such as magneto-priming (using static or electromagnetic fields), thermopriming (exposure to different temperature regimes), and treatments with gamma or laser radiation at low doses (Araújo et al., 2016). By triggering DNA repair mechanisms and modulating antioxidant enzyme activity, these treatments prime the embryo for accelerated and synchronized radicle emergence (Pagano et al., 2023). Among the physical priming methods, plasma-based treatments are emerging as promising alternatives due to the rapid processing time and low chemical input, thereby representing environmentally friendly options (Banu and Srinivasan, 2025).

Non-equilibrium gaseous plasma is a state of chemically reactive gas sustained at room temperature. This plasma contains free electrons with temperatures significantly higher (typically between 10,000 and 100,000 K) than those of the neutral gas species. The electron temperature is sufficiently high to enable frequent inelastic collisions between electrons from the high-energy tail of the distribution and gas molecules, triggering secondary chemical reactions (Mozetič, 2025). Existing literature on organic material processing predominantly employs either vacuum-based systems (1 Pa to 1 mbar) or non-equilibrium plasmas operating at ambient pressure (Holc et al., 2022). A critical distinction lies in the discharge morphology: low-pressure systems provide large-volume uniformity, while atmospheric-pressure plasmas rely on localized, transient streamers that distribute energy stochastically across the electrode gap (Mozetič, 2025). The application of plasma for seed treatment has been discussed recently (Starič et al., 2020; Holc et al., 2021; Shelar et al., 2022; Bilea et al., 2024). These treatments trigger multi-level responses, ranging from physical changes in seed coat permeability and water uptake to molecular shifts in reactive oxygen species (ROS) production, DNA integrity, and gene expression. Collectively, these mechanisms drive phenotypic and biochemical changes, ultimately improving germination kinetics and environmental resilience. In *Cannabis* plants, various cold plasma approaches have been shown to reduce fungal loads (Jerushalmi et al., 2020) and modulate terpene-related compounds (Das et al., 2025).

Owing to the multi-parametric nature of non-equilibrium plasmas and their diverse biological impacts, this study aimed to optimize hemp seed priming by systematically investigating the interplay between gas chemistry, discharge power, and treatment time. The physiological impact on seed germination was assessed by measuring germination percentage, rate, and speed. To provide a comprehensive view of the observed physiological effects, we integrated physical surface characterization (hydrophilicity and water uptake) with biochemical assays (ROS levels) and the transcriptional activation of stress defence pathways.

2. Materials and methods

2.1. Seed materials and plasma treatments

Hemp seeds of the commercial variety Futura 75 (hereafter called Futura) were used in this work. Two seed lots originating from the same production site (Rovigo, Italy) but harvested in different years were used, and are subsequently referred to as Futura 2014 (harvest year 2014) and Futura 2020 (harvest year 2020). All seeds were stored at 4° C from harvest to the moment of use.

Seeds were subjected to low-pressure non-equilibrium cold inductively coupled plasma (ICP) treatment in a semi-industrial stainless-steel reactor (Fig. S1A). Oxygen flow through the system was adjusted by FC-7700CD FCU4 flow controller (Advanced Energy Industries). The reactor was evacuated by two vacuum pumps – root (ROOTS Busch Vacuum, TYP: Wv 0500 B OHI KAFA, 600 m^3/h) and rotary (Trivac Leybold Heraeus D40B, 40 m^3/h) – connected in series. The final pressure of the system was 0.1 Pa (Lojen et al., 2022; Primc et al., 2022). This was measured by a capacitive pressure gauge (MKS Baratron pressure transducer, model number 722A11MGA2FK). Pressures of either 35 Pa or 50 Pa were used for sustaining a stable and uniform plasma in the discharge tubes. A pressure range of 30-60 Pa is optimal for sustaining a uniform plasma across the discharge tubes. At this pressure, a moderate discharge power ensures the delivery of reactive molecular radicals into the primary metallic chamber containing the seeds (Vesel et al., 2025). The luminous plasma was confined within the glass tubes, and the samples were treated with a diffusive plasma, characterized by a high density of neutral oxygen atoms in the ground state and a very low density of charged particles.

The O-atom density was measured with a calibrated catalytic probe mounted on the reactor (Fig. S1A). The probe tip was placed above the table where the seeds were assorted. The O-atom density depends on the discharge power and the pressure in the plasma reactor; all used values are presented in Table 1. The corresponding flux of O atoms was calculated using the standard kinetic equation $j = \frac{1}{4}\, n \, \langle v \rangle$, where n is the atom density and $\langle v \rangle$ the mean thermal velocity (calculated as 630 m/s at a gas temperature of 300 K). The dose (fluence) is the product of the flux and the treatment time.

For each treatment, 105 hemp seeds were treated simultaneously. Seeds were arranged in a single layer within Petri dishes and loaded onto the internal platform of the reactor vessel as shown in Fig. S1B. The reactor was evacuated to a pressure below the detection limit of the vacuum gauge (1 Pa). Then, either oxygen (O_2), hydrogen (H_2), or nitrogen (N_2) was introduced to provide the selected pressure (35 or 50 Pa) at an 800 W discharge power for different durations (0.01 s, 0.05, s 0.1 s, 1.0 s). Plasma was sustained for selected times in the E-mode. After completing the plasma treatment, the gas inlet valve was closed, the chamber was evacuated for 15-30 s to remove any residual gas, and the air was released into the chamber. Seeds were exposed to vacuum conditions for no more than 3 min. For all trials, plasma-treated seeds were compared with untreated seeds (UT).

2.2. Seed germination and physiological assays

Seed viability was assessed using the TTC (2,3,5 triphenyl tetrazolium chloride) method. Seeds were first imbibed in distilled water for 18 h to remove the achene and seed coat. Decoated seeds were incubated in a 1% TTC solution (Sigma-Aldrich, Steinheim, Germany) for 18 h at 30 °C. Each seed was then classified as viable (1), uncertain (0.5), and non-viable (0) based on the staining pattern, and the viability percentage (V%) was calculated.

Seed water uptake was monitored over a 24-h period across multiple timepoints (0, 0.5, 1, 2, 4, 6, 8, 10, 12, 16, 18, 20, and 24 h). Replicate sets (four sets of 25 seeds) were periodically removed, blotted dry, weighed, and re-imbibed to construct the imbibition curves.

Germination tests were performed following the guidelines provided by ISTA (https://www.seedtest.org/). All tests were performed with four replicates of 25 seeds each, placed in Petri dishes containing one layer of filter paper watered with 3 ml distilled water. The plates were maintained at room temperature (22 ± 2 °C) under 8/16 h light/dark photoperiod for seven days. Germinated seeds (i.e., radicle length longer than 2 mm) were counted daily. The following germination indices were

Table 1
Gas screening conditions: mode, power intensity (Watt), gas phase, flow entity (standard cubic centimeter per minute), pressure (Pascal), treatment (seconds), neutral atom density (m^{-3}), neutral atom flux ($m^{-2}s^{-1}$), neutral atom fluence (m^{-2}).

Treatment ID	Power (W)	Gas	Flow (sccm)	Pressure (Pa)	Time (s)	Neutral atom density [m^{-3}]	Neutral atom flux [$m^{-2}s^{-1}$]	Neutral atom fluence [m^{-2}]
UT	0	Air	0	-	0	-	-	-
LP	0	N_2	1155	35	180	-	-	-
N.1	800	N_2	1155	35	0.01	NA	NA	NA
N.2	800	N_2	1155	35	0.1	NA	NA	NA
N.3	800	N_2	1155	35	1	NA	NA	NA
O.1	800	O_2	1060	35	0.01	$7.17 \times 10^{20} \pm 2.06 \times 10^{19}$	$1.13 \times 10^{23} \pm 3.63 \times 10^{21}$	$1.13 \times 10^{21} \pm 3.61 \times 10^{19}$
O.2	800	O_2	1060	35	0.1	$7.17 \times 10^{20} \pm 2.06 \times 10^{19}$	$1.13 \times 10^{23} \pm 3.63 \times 10^{21}$	$1.13 \times 10^{22} \pm 3.61 \times 10^{20}$
O.3	800	O_2	1060	35	1	$7.17 \times 10^{20} \pm 2.06 \times 10^{19}$	$1.13 \times 10^{23} \pm 3.63 \times 10^{21}$	$1.13 \times 10^{23} \pm 3.61 \times 10^{21}$
H.1	800	H_2	1655	35	0.01	$2.85 \times 10^{20} \pm 4.36 \times 10^{18}$	$1.79 \times 10^{23} \pm 2.89 \times 10^{21}$	$1.79 \times 10^{21} \pm 2.89 \times 10^{19}$
H.2	800	H_2	1655	35	0.1	$2.85 \times 10^{20} \pm 4.36 \times 10^{18}$	$2.85 \times 10^{20} \pm 4.36 \times 10^{18}$	$1.79 \times 10^{22} \pm 2.89 \times 10^{20}$
H.3	800	H_2	1655	35	1	$2.85 \times 10^{20} \pm 4.36 \times 10^{18}$	$2.85 \times 10^{20} \pm 4.36 \times 10^{18}$	$1.79 \times 10^{23} \pm 2.89 \times 10^{21}$

calculated:

- G%, germination percentage: G% = 100* (N° of germinated seeds/ total N° of seeds);
- $T_{50,}$ median germination time (representative for germination seed): $T_{50} = t_i + (N/2 - n_i) \cdot [(t_i - t_j)/(n_i - n_j)]$, where N/2 is half of the total germinated seeds, n_i is the number of seeds germinated before N/2 was reached, n_j is the number of seeds germinated after N/2 was reached, t_i is the timepoint when n_i is reached, and t_j is the timepoint when n_j is reached;
- PV, peak value (representative for germination rate): $PV = (N_{max}/t_i)$, where N_{max} is the highest number of seeds germinated at day t_i, t_i is the day in which the highest number of germinated seeds was reached.

2.3. Wettability test

Wettability was measured based on the water contact angle (WCA) analysis of the seed surface. WCA was measured as described by Starič et al. (2022), immediately after the plasma treatment. For each treatment, 15 replicates of one seed each were analysed with a Drop Shape Analyzer DSA 100 E (KRÜSS GmbH, Hamburg, Germany) using 0.75 μL MilliQ (Stakpure, Niederahr, Germany) water droplets.

2.4. X-ray photoelectron spectroscopy

X-ray photoelectron spectroscopy (XPS) analyses were performed using a PHI-TFA XPS spectrometer (Physical Electronics Inc.) equipped with an Al monochromatic X-ray source. The analysed area was 0.4 mm in diameter, and the analysed depth was about 3-5 nm. The high-energy resolution XPS spectra of C 1s were acquired using an energy analyzer with a resolution of approximately 0.6 eV and a pass energy of 29 eV. During data processing, the spectra were aligned by setting the C 1s peak at 284.8 eV, characteristic of C-C/C-H bonds. Three places on the surfaces of the seed coats were analysed for every sample treatment parameter and the average surface compositions were calculated. The XPS peak areas (corrected for instrument sensitivity) were used to calculate the atomic percentages (at.%) using the formula below, where I_i represents the peak intensity (area under each peak) and S_i represents the sensitivity factor (different for each element).

$$at.\% = \frac{\frac{I_i}{S_i}}{\sum_j \frac{I_j}{S_j}} \times 100\%$$

Additionally, the O/C ratios were calculated to better evaluate the chemical changes related to these major components.

2.5. DCFH-DA assay

The DCFH-DA (2,7-dichlorofluorescin diacetate) assay was used to quantify the oxidative status of the seeds. The assay employed the fluorogenic probe DCFH-DA (Sigma-Aldrich, Milan, Italy), which undergoes intracellular deacetylation by esterases to form non-fluorescent DCFH, which is subsequently oxidized by ROS into the highly fluorescent DCF. ROS levels were determined by monitoring the fluorescence of the DCF probe, using optimal excitation/emission wavelengths of 495/529 nm. The assay was carried out as described by Pagano et al. (2022) with the following modifications. Four replicates of 10 seeds each were imbibed for 1 h in distilled water and then incubated for 30 min with 300 μL of 50 μM DCFH-DA. Subsequently, 30 μL of supernatant was used to determine fluorescence at 517 nm using a Rotor-Gene 6000 PCR apparatus (Corbett Robotics, Brisbane, Australia), with the program set for one cycle of 30 s at 25 °C. A sample containing only DCFH-DA was used as a control to subtract the baseline fluorescence. Data were expressed as relative fluorescence units (RFU).

2.6. FOX-1 assay

The concentrations of released radical peroxides [ROOH] were quantified using the FOX-1 assay as described by Griffo et al. (2023). The assay was carried out using the xylenol orange reagent (Carlo Erba, Milan, Italy), which reacts with Fe^{3+} (derived from the oxidation of Fe^{2+} induced by radical peroxides and H_2O_2) to give a blue-violet complex with maximum absorption at 560 nm. Four replicates of 10 seeds each were imbibed for 1 h in distilled water and then incubated for 45 min in FOX-1 solution containing: ammonium ferrous (II) sulphate $(NH_4)2Fe(SO_4)2 \cdot 6H_2O$ 25 mM (Merk's Reagents, Darmstadt, Germany), H_2SO_4 0, 25 M (Honeywell), xylenol orange 125 μM (Carlo Erba reagents, Milan, Italy), D-sorbitol 100 mM (Duchefa Biochemie, Haarlem, The Netherlands). The absorbance was measured at 560 nm with a Biochrom WPA Biowave (Biochrom Ltd., Cambridge, United Kingdom) spectrophotometer. To quantify [ROOH], a five-point calibration curve (0, 0.25, 0.50, 1.25, 2.50 μM) was established by mixing 2 mL of FOX-1 solution with the respective H_2O_2 standards.

2.7. RNA extraction, cDNA synthesis, and qRT-PCR

RNA was isolated from *C. sativa* treated/untreated seeds using TRIzol™ (Thermo Fisher Scientific, Monza, Italia), as indicated by the provider. Subsequently, a DNase (Thermo Fisher Scientific) treatment was performed. RNA was quantified using NanoDrop (Biowave DNA, WPA, Thermo Fisher Scientific). Following this, cDNAs were obtained by using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific) according to the manufacturer's suggestions.

To retrieve the *C. sativa* ortholog sequences of selected genes, the reference genome assembly *cs10* was used. Candidate gene sequences were identified by querying the NCBI database with the *tBLASTn* tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi), following the methodology described by Bassolino et al. (2020). The qRT-PCR reactions were performed with the Maxima SYBR Green qPCR Master Mix (Thermo Fisher

Scientific) according to the supplier's indications, by using a CFX Duet, Real-Time PCR System (BIO-RAD, Milan, Italy). Amplification conditions were as follows: denaturation at 95 °C for 10 min, and 45 cycles of 95 °C for 15 s and 60 °C for 60 s. Oligonucleotide sequences (Table S1) were designed using Primer3Plus1 (https://primer3plus.com/) and verified with Oligo Analyzer.2 (https://eu.idtdna.com/pages/tools/oligoanalyzer). Relative quantification was carried out using the F-box/-kelch-repeat protein (*F-box*) as reference gene (Deguchi et al., 2021). Raw fluorescence data provided by Software 1.7 (BIO-RAD) were used to determine the threshold cycle number (Ct) values for each transcript quantification. Relative quantification of transcript accumulation was performed as described in Thomsen et al. (2010). All reactions were carried out in triplicate. For data representation, Z-score was calculated on the linearized Ct values and used to generate a heatmap.

2.8. Statistical analysis

One-way ANOVA was applied throughout the study. The Student's *t*-test (*, *p*-value ≤ 0.05) was applied to compare untreated with treated seeds, or to compare seeds belonging to the same variety, when considering morphological and physico-chemical analyses. Pearson correlation analyses were performed with the *agricolae* and *r-statix* packages in the R background (Kassambara, 2019).

3. Results

3.1. Screening for ICP conditions based on germination effects

Prior to optimizing ICP priming, the seed quality of two Futura 75 lots was characterized. This was assessed by evaluating the final germination percentage (G%), and viability (V%) as determined by TTC staining. The Futura 2020 seed lot presented significantly higher G% (79.0 ± 3.3 %) and V% (94.4 ± 6.0 %) compared to Futura 2014 (52.0 ± 8.2 G%; 79.2 ± 2.4 V%) (Fig. S2). A notable discrepancy was observed within the Futura 2014 lot, where the V% substantially exceeded the G %, suggesting a high proportion of dormant or low-vigour seeds. This suggests that while a portion of the seeds remains metabolically active, their failure to germinate may be attributed to either deep dormancy or a loss of vigour associated with seed aging.

To investigate the effect of different gas sources, both seed lots were exposed to plasma generated using N_2, O_2, and H_2 at three exposure times (0.01, 0.1, 1.0 s). Initially, to ensure consistency across the experimental groups, a constant working gas pressure of 35 Pa was maintained for all treatments. PV and T_{50} were used as screening indices as they provide a more dynamic measure of germination speed and vigour compared to G% alone. For the Futura 2014 seed lot, none of the applied treatments improved germination; in fact, certain conditions (e.g., LP, N.2, O.3) exerted a detrimental effect (Fig. 1a). When considering the Futura 2020 seed lot, positive effects were observed when using the O.1 and O.2 treatments which significantly increased the germination rate (in terms of PV values) compared to untreated seeds (Fig. 1b). These findings are further supported by the daily G% measurements, which reveal enhanced performance for the O.1 and O.2 treatments in Futura (2020), whereas the Futura 2014 lot failed to respond positively (Fig. S3).

Given the detrimental impact of the 35 Pa vacuum conditions (LP) on the Futura 2014 lot, subsequent screenings were conducted at higher pressures of 50 and 65 Pa. Unlike the 35 Pa, both conditions were non-inhibitory (Fig. 2a), so we further selected 50 Pa to maintain a lower operating pressure, which typically facilitates a more stable plasma discharge. To finalize the treatment selection, oxygen plasma was applied at the specific pressures optimized for each seed lot, with the inclusion of an additional exposure time (0.05 s) to better resolve the transition between the 0.01 and 0.1 s treatments. All treatment combinations are provided in Table S2. While most treatments (except 1.0 s) enhanced the germination kinetics of Futura (2020) (blue bars), the

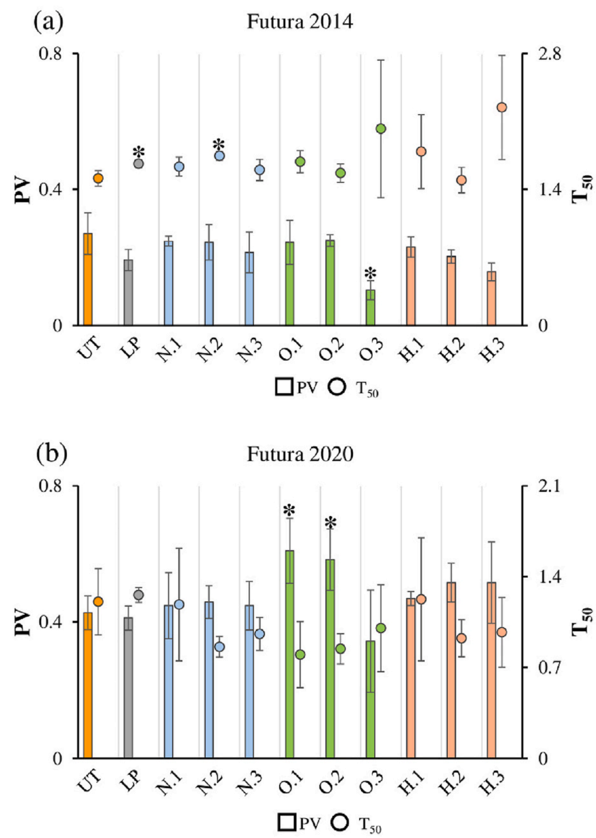

Fig. 1. Screening for ICP gas phase. Peak value (PV) and median germination time (T_{50}) in **(a)** Futura 2014 and **(b)** Futura 2020 seeds subjected to plasma treatments. Data represent the mean ± standard deviation of four independent replicates. Each treatment was compared to the untreated control (UT), and statistically significant differences, as per the Student's *t*-test, are indicated by asterisks (*; $p \leq 0.05$). All abbreviations are hereby explained: N.1, 0.01 s nitrogen plasma; N.2, 0.1 s nitrogen plasma; N.3, 1.0 s nitrogen plasma; O.1, 0.01 s oxygen plasma; O.2, 0.1 s oxygen plasma; O.3, 1.0 s oxygen plasma; H.1, 0.01 s hydrogen plasma; H.2, 0.1 s hydrogen plasma; H.3, 1.0 s hydrogen plasma; LP, low pressure (35 Pa); UT, untreated seeds.

exposure to 0.1 s and 1.0 s was still detrimental to the Futura 2014 lot (orange bars), as reflected by the low PV values (Fig. 2b).

These screening results revealed a divergence between the two seed lots: while the 0.1 s treatment was beneficial for the Futura 2020 seed lot, the same exposure time negatively impacted the Futura 2014 seeds. Adjustments to both working pressure and treatment duration failed to produce a positive effect in the Futura 2014 seed lot. Notably, exposure to 1.0 s was detrimental also for the Futura 2020 lot.

3.2. Plasma treatments change hemp seed chemical composition and wettability levels

To investigate the mechanisms underlying the improved germination in Futura (2020), the surface elemental composition was characterized via X-ray photoelectron spectroscopy (XPS). This analysis evaluated all gas phases at the 0.1 s exposure time (identified as the most effective duration for the Futura, 2020 lot) to provide a comparative baseline and elucidate the chemical surface modifications unique to the oxygen treatment (Table 2). The survey spectra indicate that the seed surface is primarily composed of carbon and oxygen, consistent with the presence of an organic, carbon-rich layer such as the lignocellulosic seed coat.

After O_2 plasma treatment, the increase in oxygen content (O at%, Table 2) reflects the formation of oxidized functional groups such as hydroxyl (C-O/C-OH), carbonyl (C=O/O-C-O), or carboxyl (O-C=O)

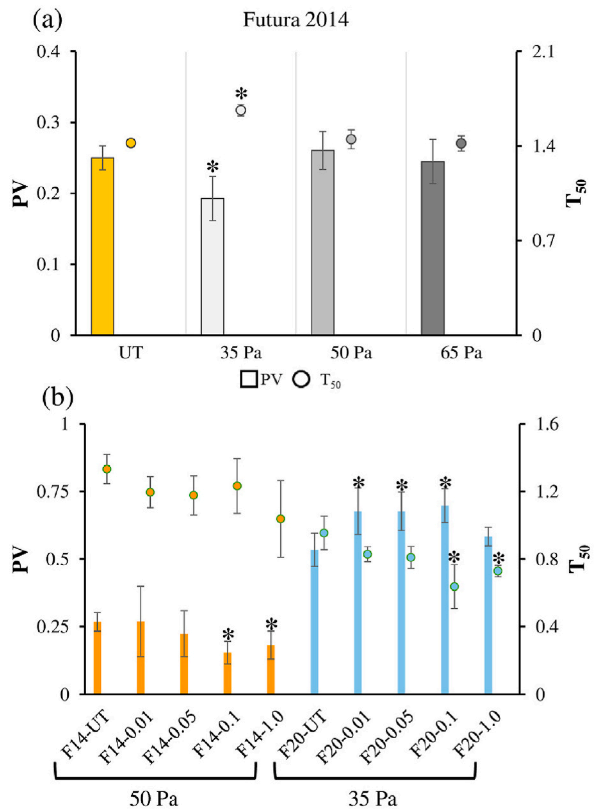

Fig. 2. Screening for oxygen plasma pressure and time exposure. **(a)** Peak value (PV) and median germination time (T_{50}) in the Futura 2014 seed lot subjected to different pressures of 35, 50, and 65 Pa. **(b)** Peak value (PV) and median germination time (T50) in the Futura 2014 (F14) and Futura 202 (F20) exposed to different pressures and exposure times: 0.01 s, 0.05 s, 0.1 s, and 1.0 s. Data represent the mean ± standard deviation of four independent replicates. Each treatment was compared to the untreated control (UT), and statistically significant differences, determined using the Student's *t*-test, are indicated by asterisks (*; $p \leq 0.05$).

species, which enhance the surface hydrophilicity and chemical reactivity. This also led to a decrease in the carbon content (C at%), indicating that the carbonaceous surface layers were oxidized and possibly partially removed by the plasma treatment. The N_2 and H_2 plasma treated samples showed only minor surface changes compared to untreated seeds, with about 1% decrease in carbon and a similar increase in oxygen. Minor signals for silicon (Si), calcium (Ca), and N were also identified. While the N signal likely stems from the adsorption of nitrogenous atmospheric species upon exposure to ambient air, the detection of Si and Ca is common in seed coat analyses, as these elements are often localized within the outermost layers of the testa (Holc et al., 2022).

To quantify the treatment's impact, the O/C ratio was calculated; this ratio doubled from 0.1 in UT to 0.2 after the O_2 plasma treatment, indicating enhanced surface oxidation. Deconvolution of the C 1s XPS spectra (Fig. 3a and b) illustrates the surface modification process: O_2 plasma exposure for 0.1 s reduced the dominance of non-polar C-C/C-H bonds while significantly increasing the concentration of oxygen-containing functional groups (C-O/C-OH, C=O, O=C-O).

To further investigate the relationship between surface modification and water uptake, WCA measurements were performed on both seed lots across all O_2 plasma exposure times (Fig. 3c). This allowed for a comparative assessment of how surface wettability changes influenced the divergent germination responses. WCA is a measure of the interaction between a water droplet and a solid surface, representing the wettability in terms of hydrophobicity or hydrophilicity. All treatments resulted in a reduction of the WCA values, with the longest treatment (1.0 s) having the highest impact, thereby achieving maximal levels of hydrophilicity. This may be explained by the introduction of oxygen-containing functional groups, as indicated by the XPS analysis.

3.3. Plasma overtreatment triggers excessive water uptake and oxidative stress

To elucidate the divergent germination patterns observed between short (0.1 s) and long (1.0 s) O_2 plasma exposures, the oxidative status and water uptake dynamics were investigated across both seed lots. The oxidative status was assessed by quantifying the accumulation and release of ROS from both plasma-treated and control seeds (Fig. 4). A significant increase in the release of peroxide radicals (Fig. 4a) was observed in both seed lots only after the long exposure time (1.0 s), while ROS accumulation was unaffected (Fig. 4b). This may indicate that the long exposure time favors ROS release rather than accumulation in seeds. We hypothesize that this may be due to high oxidative stress, possibly as a direct consequence of the overly rapid rehydration observed in the WCA.

To test this hypothesis, the imbibition curves of Futura (2020) seeds following exposure to 0.1 s and 1.0 s were determined (Fig. S4). Both treatments showed higher water uptake than the control throughout the first 14 h of imbibition. A significant variation was observed at 0.5 h (30 min), where the 1.0 s exposure (0.114 ± 0.004 g) induced a more rapid initial hydration than the 0.1 s (0.091 ± 0.007 g). Collectively, these findings suggest that the high oxidative stress associated with the 1.0 s exposure may be a secondary effect of accelerated imbibition, where the extreme hydrophilicity of the seed coat leads to imbibitional damage.

3.4. Plasma treatments induce changes in gene expression patterns in seeds

Building upon the physical, chemical and physiological findings, a transcriptional analysis via qRT-PCR was conducted to investigate the molecular mechanisms triggered by O_2 plasma treatment. To pinpoint the molecular factors responsible for the observed germination enhancement, this analysis focused specifically on the Futura 2020 seeds treated for 0.1 s, as this condition represented the optimal priming effect.

The expression profiles of a panel of target genes, including *Cu/Zn SOD* (superoxide dismutase [Cu-Zn]), *RbohD* (respiratory burst oxidase homolog D), *E2FA* (elongation transcription factor 2 A), *CD3.3* (cyclin

Table 2
Surface chemical composition in atomic percentage (at. %) derived from the XPS spectra. The relative concentrations (%) of different bonds of carbon atoms on the surface are also indicated. The treatment time in plasmas was 0.1 s. Each treatment was compared to the untreated control (UT), and statistically significant differences, determined using the Student's *t*-test, are indicated by asterisks (*; $p \leq 0.05$).

							C-C/C-H	C-O/C-OH	C=O/O-C-O	O-C=O
Sample	C (at%)	O (at%)	Si (at%)	Ca (at%)	N (at%)	O/C	284.8 eV	286.4 eV	287.8 eV	289.1 eV
F20-UT	89.5 ± 0.8	8.9 ± 0.7	0.8 ± 0.3	0.4 ± 0.1	0.4 ± 0.2	0.100	93.6	3.4	1.1	1.9
O_2	80.6 ± 1.2*	16.9 ± 0.4*	1.5 ± 0.5	0.5 ± 0.1	0.6 ± 0.4	0.209	82.2	10.6	2.8	4.4
N_2	87.2 ± 0.5	10.6 ± 0.3	1.8 ± 0.3*	0.4 ± 0.1	0	0.122	91.7	5.7	0.4	2.2
H_2	88.9 ± 0.5	9.8 ± 0.2	1 ± 0.2*	0.4 ± 0.1	0	0.111	89.5	7.3	1.5	1.7

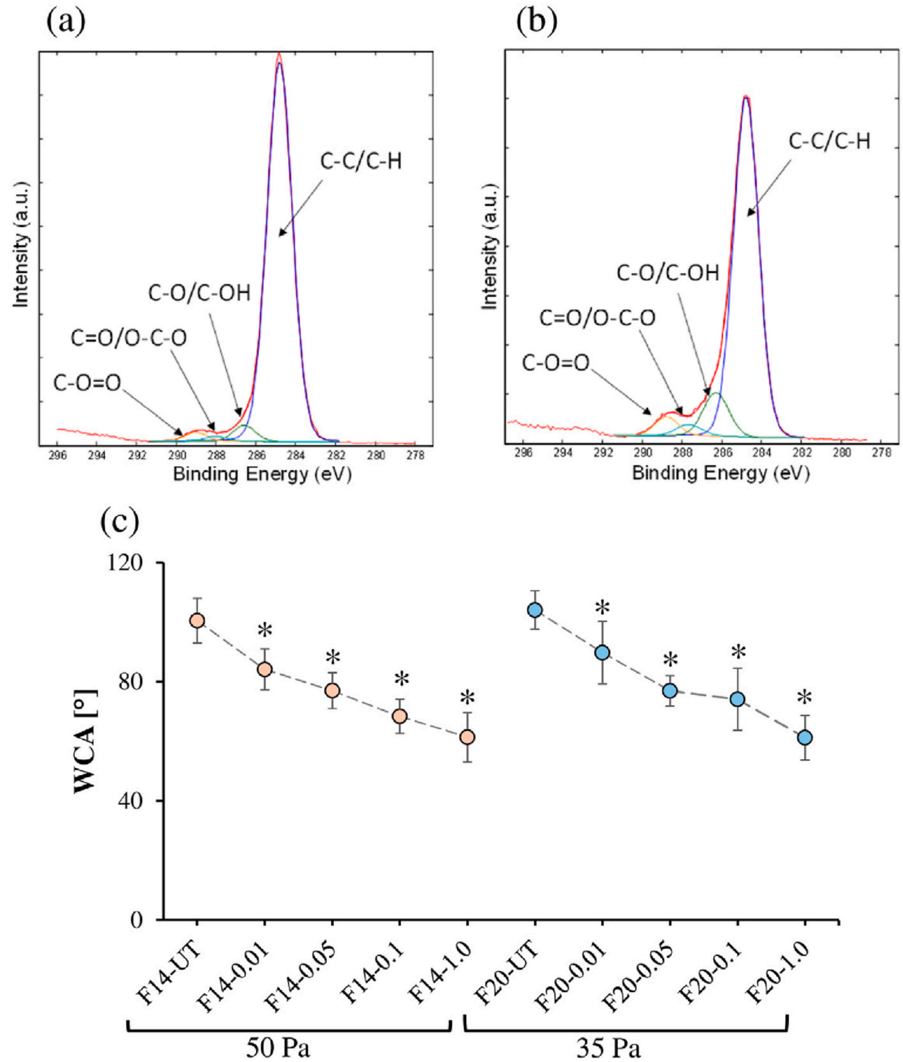

Fig. 3. Elemental composition and wettability measurements. High-resolution X-ray photoelectron spectroscopy (XPS) spectra of the C 1s region for **(a)** the untreated (UT) and **(b)** O_2 plasma-treated seeds of Futura (2020). The C 1s spectra reveal the chemical states of carbon present on the surface before and after plasma exposure, enabling comparison of surface functionalization and oxidation induced by the O_2 plasma treatment. **(c)** Water contact angle (WCA) measurements in the Futura 2014; Futura 2020 seed lots following exposure to O_2 plasma at different timepoints and pressures. Data represent the mean ± standard deviation of four independent replicates. Each treatment was compared to the untreated control (UT), and statistically significant differences, determined using the Student's *t*-test, are indicated by asterisks (*; $p \leq 0.05$).

D3.3), *CKI7* (cyclin kinase inhibitor 7), and *TDP1* (tyrosyl DNA phosphodiesterase 1) were investigated. While *Cu/Zn SOD* is involved in the removal of superoxide radicals, *RbohD* is responsible for the production of ROS (Nadarajah, 2020). *E2FA* acts as a transcription factor promoting cell proliferation in early developmental stages, whereas *CD3.3* controls the G_1-S phase transition of the cell cycle, and *CKI7* serves as a negative regulator of cyclin-dependent activity (Boudolf et al., 2004). The *TDP1* gene encodes for a DNA repair enzyme participating in the removal of DNA-protein crosslinks, envisioned as a seed quality hallmark (Forti et al., 2020; Pagano et al., 2017). This specific panel of target genes was selected to provide a snapshot of the molecular shifts targeting three critical pathways, namely redox homeostasis (*Cu/Zn SOD, RbohD*), cell cycle activation (*E2FA, CD3.3, CK7*), and DNA repair efficiency (*TDP1*). The coordinated regulation of these pathways is important for maintaining seed vigour, as they orchestrate the transition from metabolic dormancy to active embryo growth while mitigating oxidative damage during imbibition (Gualtieri et al., 2021).

To investigate the transcriptional shifts associated with plasma-induced water dynamics, we monitored the expression of these genes in dry seeds (0 h) and during imbibition at 12 h and 24 h. The relative expression data are presented in Table S3, while a heatmap utilizing Z-score normalization was constructed to facilitate the visualization of transcriptional patterns (Fig. 5). The data showed that the *CD3.3, RbohD, Cu/Zn SOD* and *TDP1* genes were significantly upregulated in the dry seeds exposed to plasma. The highest relative expression was observed for the *CD3.3* cell cycle promoter which remained upregulated also at 12 h during imbibition. At 24 h, all genes were highly expressed in both treated and untreaded seeds as this timepoint represent the transition towards radicle protrusion and the activation of the post-germinative growth program. Within the plasma treated seeds, the *CD3.3* and *Cu/Zn SOD* genes were down-regulated, possibly indicating an advanced developmental state.

Overall, upregulation of target genes in dry seeds followed by downregulation at 24 h may indicate that plasma treatment effectively triggers germination also at the molecular level, allowing the seeds to complete their pre-germinative metabolic requirements sooner than untreated controls.

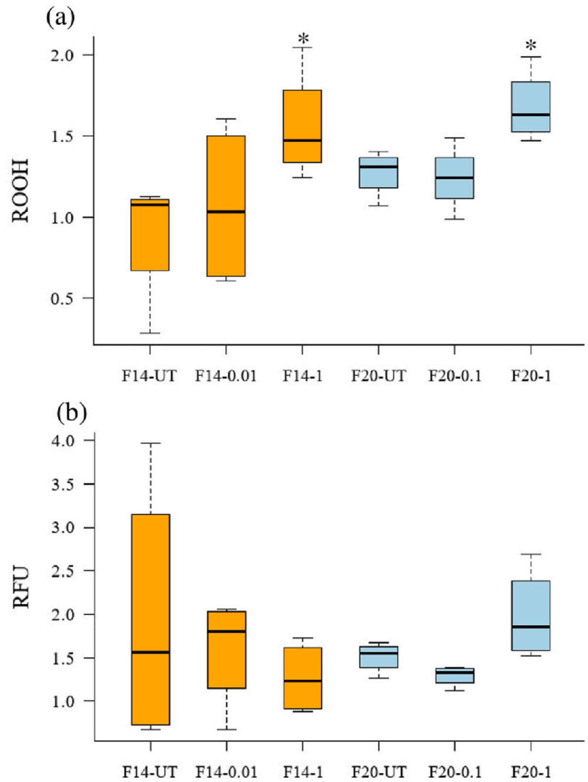

Fig. 4. Oxidative status of 2014 and 2020 Futura 75 seed lots exposed to short (0.1 s) and long (1 s) O_2 plasma exposure. **(a)** Concentration of radical peroxide [ROOH] released from seeds. **(b)** ROS accumulation in terms of relative fluorescence units (RFU). Data represent the mean ± standard deviation of four independent replicates. Each treatment was compared to the untreated control (UT) and statistically significant differences, determined using the Student's *t*-test, are indicated by asterisks (*; $p \leq 0.05$).

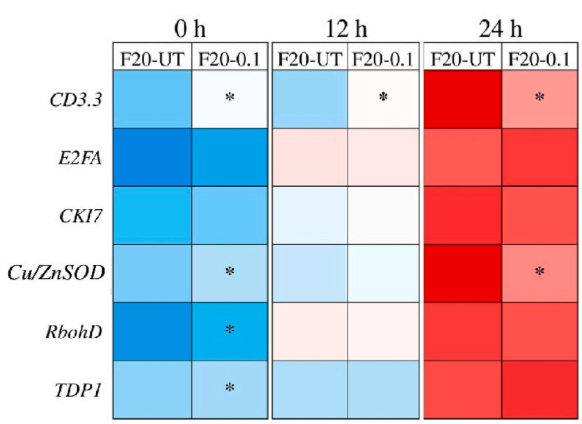

Fig. 5. Heatmaps representing the relative expression of selected genes in the Futura 2020 seed lot exposed to O_2 plasma treatments for 0.1 s. Data were gathered from dry seeds (0 h) and from imbibed seeds at 12 and 24 h. Data represent the mean of three replicates. Each treatment was compared to the respective untreated control (UT), and statistically significant differences, determined using the Student's *t*-test, are indicated by asterisks (*; $p \leq 0.05$). *CD3.3*, cyclin D3,3; *E2FA*, transcription elongation factor 2 A; *CKI7*, cyclin-dependent kinase inhibitor 7; *Cu/Zn SOD*, superoxide dismutase [Cu-Zn]; *RbohD*, respiratory burst oxidase homolog protein D; *TDP1*, tyrosyl-DNA phosphodiesterase 1.

3.5. Gene expression correlates with hydrophilicity and germination

Correlation analyses were performed to integrate the datasets regarding germination performance, surface hydrophilicity, and transcript profiles, providing a holistic view of the plasma-seed interactions (Fig. 6). The results showed that WCA negatively correlated with the gene expression data and germination rate (PV). Conversely, a positive correlation was observed between WCA and T_{50}, confirming that increased surface hydrophilicity directly translates to accelerated germination speed. Reflecting an overall activation of the pre-germinative program, PV was positively associated with the majority of the gene panel, whereas T_{50} showed an inverse relation with most transcripts, confirming that higher expression levels generally led to faster and more successful germination. Intra-gene correlation analysis revealed a highly coordinated transcriptional network in dry seeds, where *CD3.3* was positively associated with *Cu/Zn SOD*, *RbohD*, and *TDP1*, reflecting a synchronized activation of the antioxidant, signalling, and repair pathways.

Overall, these data further support the finding that plasma priming applied to Futura 2020 for 0.1 s results in increased hydrophilicity and enhanced transcription of investigated genes, ultimately leading to faster and more uniform germination.

4. Discussion

A successful cultivation of industrial hemp depends on high and uniform seed germination. Research has established several key environmental (e.g., temperature, water availability, storage conditions) and intrinsic (e.g., maturity state, dormancy, genetic background) factors influencing this initial stage (Sorokin et al., 2021). The mechanisms of seed dormancy and factors causing the decline in viability during storage are not fully understood; therefore, more research underlining biochemical and molecular characterization of hemp seed quality and germination aspects are encouraged. Moreover, to enhance seed quality, pre-sowing treatments can be applied. The application of priming approaches on *C. sativa* seeds is of recent interest and few authors have reported some improvements in germination following stress conditions

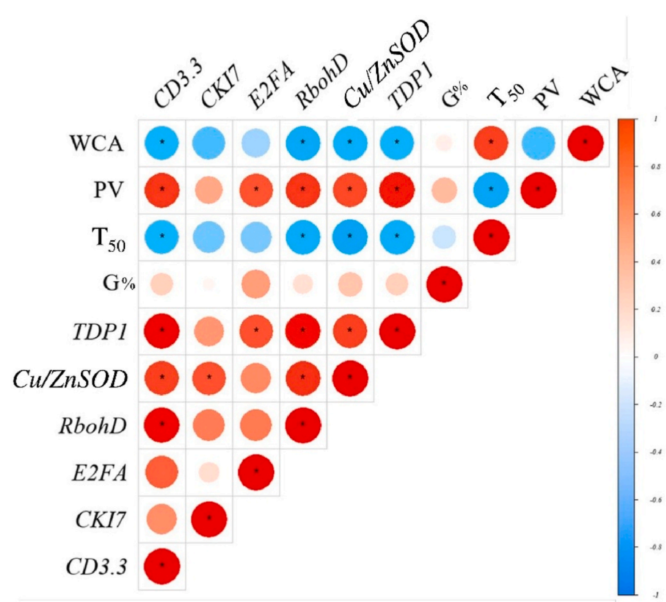

Fig. 6. Pearson correlation indices calculated for oxygen plasma-treated Futura 2020 seeds. Data used included germination parameters (G%, PV, T_{50}), wettability (WCA), and gene expression profiles (*CD3.3, E2FA, CKI7, Cu/Zn SOD, RbohD, TDP1*) in dry seeds. In blue are given the negative correlations, while red colour represents positive correlations. Statistically significant correlations are indicated by asterisks (*; $p \leq 0.05$).

in response to hydropriming, halopriming, hormopriming, or osmopriming (Ghasempour et al., 2024; Kasprowiak et al., 2025; Latif et al., 2025). However, these chemical-based priming techniques are often conditioned by the genetic background. Alternatively, physical priming could represent an efficient and often environmentally friendly strategy. In this sense, plasma treatments are gaining increasing interest from both the research community and farmers, even though treatment optimization is affected by a multitude of parameters, which need to be tested individually (Waskow et al., 2021). So far, cold plasma has been tested in *Cannabis* seeds only in a few studies (Sera et al., 2017; Iranbakhsh et al., 2020; Ghasempour et al., 2024). Seeds treated with Gliding Arc plasma (Sera et al., 2017) or Dielectric Barrier Discharge plasma (Iranbakhsh et al., 2020) presented different degrees of improvements in germination and seedling growth. Despite these advances, the physicochemical and molecular mechanisms underlying plasma priming in hemp seeds remain poorly understood.

Accordingly, in this study, we tested various parameters (e.g., gas composition, pressure, exposure time) to identify optimal ICP treatments and elucidate the underlying causes of the resulting effects on hemp seeds. To achieve this, we utilized two seed lots of Futura 75, one of the most widely cultivated and versatile industrial hemp varieties in Europe, primarily used for fibre, seed production, and CBD extraction, with THC levels of less than 0.2% (Ascrizzi et al., 2024). These seed lots exhibited substantial differences in terms of germination percentage, but less so in terms of viability (Fig. S1). This indicates that the Futura 2014 lot, presenting high V% but low G%, displayed dormancy-related traits whereas the 2020 lot did not, given its similar G% and V% values. Alternatively, the Futura 2014 seeds may have undergone age-related deterioration, despite being continuously stored at 4° C prior to use.

The first step in optimizing ICP treatments was testing different gases, namely N_2, O_2, and H_2. The germination screening results indicated that O_2-generated plasma was the most efficient treatment, but only for the Futura 2020 seed lot and at specific exposure times (Fig. 1b). Oxygen plasma is known to facilitate surface oxidation of organic matter via ground-state atomic oxygen, which induces irreversible functionalization. While hydroxyl saturation occurs at low doses (cca. 10^{20} m^{-2}), the formation of more complex oxygen-containing functional groups requires significantly higher radical fluences (Polito et al., 2022; Zaplotnik and Mozetič, 2022). Upon exposure to high-density O_2 plasma, seeds rapidly attain a super-hydrophilic state as the surface functionalization threshold is reached within a sub-second timeframe (Vesel et al., 2025). Indeed, this is in agreement with our results regarding the XPS spectra and WCA measurements conducted following a 0.1 s exposure time (Fig. 3), indicating the formation of oxidized functional groups and enhanced wettability. However, longer treatment times have been associated with etching of organic matter and destruction of the original surface structure (Primc, 2025). The attribution of increased wettability of hydrocarbons to the formation of additional polar functional groups may not always be justified. Namely, reactive species from oxygen plasma cause etching, which is often non-homogeneous, leading to nanostructuring after prolonged treatment. The selected flux of oxygen atoms and the short treatment times, however, make these effects marginal. While the etching rate of epicuticular wax by atomic oxygen remains uncharacterized at room temperature, data from polymer films provide a useful benchmark. Zaplotnik et al. (2016) reported an etching rate of 0.01 nm/s at an O-atom density of 1.2×10^{21} m^{-3}. Although waxes may etch more readily than synthetic polymers, the sub-second treatment times (less than 1 s) employed here likely preclude significant material loss.

Indeed, in our experiments, only the long exposure time (1.0 s) resulted to be damaging to the germination process in both seed lots (Fig. 2b). Moreover, this overtreatment resulted in higher ROS release from seeds during imbibition, potentially linked with increased oxidative stress (Fig. 4). In fact, the imbibition process *per se* can result in cellular damages and membrane rearrangements that in turn can affect ROS accumulation or leakage (Doria et al., 2019). Another interesting finding is that the 1.0 exposure resulted in an over-intensified water uptake in the first 30 min of imbibition compared to the 0.1 s (Fig. S4). Overall, these results suggest that excessive water uptake in seeds exposed to longer treatments may have caused cellular damage, ultimately reducing germination performance. In this sense, we propose the hypothesis of a “hydrophilicity window”; within its range, germination is enhanced, whereas exceeding it reduces germination performance due to excessive water uptake and consequent cellular damage. A detrimental effect of prolonged treatments was observed also in wheat seeds subjected to glow plasma treatment for 30 and 90 s (Starič et al., 2022). The authors linked it to increased surface roughness, higher concentrations of elements (oxygen, potassium, sulfur, calcium, and magnesium), along with exacerbated water uptake.

Another finding worth discussing refers to the fact that none of the plasma treatments improved germination in the Futura 2014 lot, likely due to the presence of dormancy or seed ageing related deterioration. This is in line with other studies where the efficiency of plasma-priming applied to sixty *Trifolium pratense* seed lots depended on the initial seed quality and was constrained by dormancy variations linked to harvest year, developmental conditions, and storage time (Kavka et al., 2025). While oxygen plasma may overcome physical dormancy by increasing surface hydrophilicity and eroding the epicuticular wax, it cannot act on internal age-related deterioration (Cui et al., 2019). For aged, dormant hemp seeds, the treatment does improve water uptake, but may fail to trigger the metabolic transition from quiescence to active growth. Consequently, the seeds become super-hydrophilic without achieving viability, as the plasma acts only on the surface and cannot aid to repair degraded materials.

Finally, to investigate the positive effects of ICP treatments at the molecular level, the transcriptional levels of specific genes involved in ROS homeostasis (*Cu/Zn SOD, RbohD*), DNA repair (*TDP1*), and cell cycle regulation (*E2FA, CD3.3, CKI7*) were measured. These processes were targeted as they are known to be related to seed quality traits in different species (Pagano et al., 2017; Forti et al., 2020; Gualtieri et al., 2021). Our results indicate that the majority of genes were upregulated in treated seeds at the dry stage while some appeared to be downregulated at later stages (Fig. 5). This suggests that this specific treatment improves seed quality and subsequent germination performance through the early induction of relevant transcriptional pathways. When correlation analyses were conducted, WCA showed a positive correlation with faster germination (T_{50}) and gene expression data (Fig. 6). This further indicate that plasma treatments modulates both germination performance and transcriptional activity. Moreover, a sustained effect of the positive treatments was observed, with the persisting upregulation of the *CD3.3* gene at 12 h during imbibition. During germination, cyclins D help trigger the onset of cell division in embryonic tissues, enabling radicle emergence and early seedling growth. By integrating environmental and hormonal signals, cyclins D ensure that cells resume proliferation only when conditions are favourable for a successful germination (Vázquez-Ramos and de la Paz Sánchez, 2003; Lara-Núñez et al., 2008). However, the gene was downregulated at 24 h during imbibition, a period marking the transition into post-germinative metabolism. This transcriptomic shift may suggest that the primary phase of cell cycle activation (G1 to S transition) is replaced by seedling establishment processes, where cellular resources are reallocated from mitotic preparation to radicle elongation and nutrient mobilization, in agreement with existing literature (Masubelele et al., 2005).

5. Conclusion

The present study demonstrated that O_2-induced plasma treatments, delivered at low exposure time, effectively enhanced the germination performance of the Futura 75 non-dormant seed lot.

The observed improvements originate from a dual-action process enabled by the high O-atom flux of a low-pressure O_2 plasma. This configuration ensures rapid modification kinetics that simultaneously alter the seed's chemical and physical surface properties. The short exposure time (0.1 s) elicited a priming response characterized by the induction of pre-germinative metabolic pathways and antioxidant systems. These changes are mediated by specific signalling cascades preparing the seed to subsequent germination cues. As evidenced by the XPS and WCA analyses, the plasma treatment induced rapid changes in the seed elemental composition and surface hydrophilicity, facilitating higher water uptake and subsequent acceleration of metabolic activity. However, at prolonged exposure times (1.0 s), a detrimental effect is evident, leading to the hypothesis that a "hydrophilicity window" may exist; this is characterized by excessive water uptake, correlated with high oxidative damage and reduced germinability. Lastly, none of the tested treatments could improve the germination performance in the Futura 2014 seed lot, suggesting that dormancy or aging-related processes act as critical bottlenecks that circumvent the priming effects of oxygen plasma. Future research should evaluate the biochemical thresholds of plasma-induced oxidative stress on pre-germinative metabolism to identify the transition point between beneficial and detrimental effects.

The scalability of ICP seed priming may facilitate its integration into industrial agricultural workflows. By utilizing a vertical transit system, seeds can be processed at high speeds (e.g., 0.1 s exposure) as they pass through the discharge zone. This method guarantees a uniform distribution of surface functionalization, making it an economically viable alternative to chemical-based priming.

Author contributions

AM and MM conceptualized the study. NB, DL, and NA performed the experiments and analysed the data; namely, DL and RZ was involved in developing the CP treatments while NB and NA also performed the phenotyping and molecular analyses. DL and JK performed the XPS analysis. MM was responsible for seed production, collection and storing. LB and RP participated in funding acquisition and manuscript drafting. AM, NB, MM, and AB drafted the manuscript. All authors were involved in reviewing and editing the manuscript.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2026.111268.

Data availability

Data will be made available on request.

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