Innovative Food Science and Emerging Technologies 49 (2018) 82–91

Contents lists available at ScienceDirect

Innovative Food Science and Emerging Technologies

journal homepage: www.elsevier.com/locate/ifset

Effects of non–thermal plasmas on seed-borne *Diaporthe/Phomopsis* complex and germination parameters of soybean seeds

María Cecilia Pérez Pizá[a], Leandro Prevosto[b], Carla Zilli[a], Ezequiel Cejas[c], Héctor Kelly[c], Karina Balestrasse[a,*]

[a] Instituto de Investigaciones en Biociencias Agrícolas y Ambientales (INBA), Facultad de Agronomía, Universidad de Buenos Aires (UBA), Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), San Martín 4453, Buenos Aires, Argentina
[b] Grupo de Descargas Eléctricas, Departamento Ing. Electromecánica, Facultad Regional Venado Tuerto (UTN), Laprida 651, Venado Tuerto, Santa Fe, Argentina
[c] Universidad Tecnológica Nacional, CONICET, Facultad Regional Venado Tuerto, Departamento de Ingeniería Electromecánica, Grupo de Descargas Eléctricas, Laprida 651, Venado Tuerto, Santa Fe, Argentina

ARTICLE INFO

Keywords:
Non-thermal plasma
Diaporthe/Phomopsis complex
Glycine max
Seed quality

ABSTRACT

Diaporthe/Phomopsis (D/P) is a complex of seed-borne fungi that severely affects soybean (*Glycine max* (L.) Merrill), one of the most important crops worldwide. Non-thermal plasma treatment is a fast, economic and ecological friendly technology that can destroy seed-borne fungi and improve seed quality. Soybean seeds were exposed for 1, 2 and 3 min to a quasi-stationary (50 Hz) dielectric barrier discharge plasma operating at atmospheric pressure air. Different carrying gases (O_2 and N_2) and barrier-insulating materials were used. In this work we focused on the ability of plasma to control D/P in soybean seeds and to enhance seed quality. To support these results, different antioxidant enzymes (catalase, superoxide dismutase and guaiacol peroxidase), lipid peroxidation and phytohormones (ABA and AIA) content in seeds were evaluated. The results demonstrated reductions of 29% in catalase activity and increments of 30% in glutathione content after plasma treatment, reversing the oxidative damage caused by D/P fungi. This eco-friendly technology improved soybean seed quality and, for the first time, its efficiency in controlling soybean seed-borne pathogen fungi that colonize the inside of seeds was demonstrated.

1. Introduction

Soybean (*Glycine max* (L.) Merrill) is one of the most important crops worldwide. In Argentina, the production of soybean and its derivatives is one of the most dynamic economic activities and it has been increasing throughout the years. In the last growing season (2016–2017) soybean sown area reached 19 million of hectares and the production yield was 57 million metric tons (Terré, 2018). Seed quality is one of the principal factors that must be considered when sowing as it guarantees plant growth and development and crop yield. High temperatures and relative humidity that have characterized the last harvest seasons contributed to the intensification of pathogens affecting crops, making difficult to produce high quality seeds.

The *Diaporthe/Phomopsis* (D/P) complex is one of the most harmful group of fungal pathogens in soybean and includes the seed-borne causal agents of soybean stem canker (*Diaporthe phaseolorum* var. *caulivora* and *D. aspalathi*) and pod and stem blight and seed decay (*D. phaseolorum* var. *sojae* and *Phomopsis loingicolla*) (Sánchez, Ridao, & Colavita, 2015). In addition to the great yield losses that this complex provokes, infected seeds are often responsible for the spread of the diseases to new areas (Singh & Mathur, 2004). The use of chemical fungicides for seed treatment as a method for protecting the crop in its early development stages is one of the most common practices between farmers. Agrochemicals are known to remain in harvested products and in the fields disrupting the natural state of the microbiological biota and contaminating the soil and water in agricultural areas (Carvalho, 2006). Historically, the intensive use of fungicides has led to the appearance of resistant fungal strains (Hahn, 2014). In this scene, non-thermal plasmas arise as a novel approach to fungal pathogen control in seeds as they represent a fast, economic and eco-friendly technology.

Non-thermal plasmas are (quasi-neutral) partially ionized gases usually generated by low-current electrical discharges. They are constituted by molecules, atoms, free radicals, ions, reactive species, high-energy electrons, ultraviolet (UV) photons and high-electric fields (Misra, Schlüter, & Cullen, 2016). As most of the energy is carried by the electrons, the ions and neutrals remain relatively cold resulting in a

* Corresponding author at: Facultad de Agronomía, Universidad de Buenos Aires, San Martín 4453, 1417 Buenos Aires, Argentina.
E-mail address: kbale@agro.uba.ar (K. Balestrasse).

https://doi.org/10.1016/j.ifset.2018.07.009
Received 11 April 2018; Received in revised form 11 July 2018; Accepted 12 July 2018
Available online 20 July 2018

non-thermal plasma (or cold plasma) at atmosphere pressure that can be sprayed onto heat-sensitive surfaces including biological tissues (Laroussi, 2005).

Among different kind of non-thermal atmospheric-pressure plasma sources, the dielectric barrier discharges (DBD) have a large number of technological applications because they operate at strongly non-equilibrium conditions at atmospheric pressure and at reasonably high-power levels. It is based on the use of a dielectric barrier in the discharge gap, which stops electric current and prevents spark formation (Brandenburg, 2017). The presence of a dielectric barrier precludes direct-current operation of DBD, which usually operates at frequencies between 0.05 and 500 kHz. When the electric field in the discharge gap is high enough to cause breakdown, in most gases, a large number of plasma filaments (or microdischarges) are observed when the pressure is of the order of the atmospheric value. In this filamentary mode discharge, plasma formation resulting in electrical conductivity, are restricted to the plasma filaments. Under certain circumstances, also apparently homogeneous (diffuse) discharges can be obtained. This characteristic depends on the operating conditions, such as electrode and dielectric barrier configurations, gas mixture, gap distance, frequency and voltage. The highly reactive species as well as UV radiation are considered to be the most important inactivation agents in plasma as they induced oxidative stress that cause cell damage and cell death (Fridman et al., 2008; Laroussi & Leipold, 2004; Zhang et al., 2012). Plasma treatments have showed to induce multiple mechanisms of action and the biological reactions range from intercellular DNA fracture and protein degeneration to oxidation of the outer membrane (Ma, Cholewa, Mohamed, Peterson, & Gijzen, 2004; Moisan et al., 2001). Furthermore, as multiple mechanisms of action are involved, the evolution of microbial resistance is counteracted (Shama & Kong, 2012). Many experiments found that non-thermal plasmas can inhibit the growth of clinical fungi (Daeschlein et al., 2014; Sun et al., 2011), food contaminant fungi (Basaran, Basaran-Akgul, & Oksuz, 2008; Dasan, Mutlu, & Boyaci, 2016) and phytopathogenic fungi (Zhang et al., 2014). On the other hand, several works have showed that plasma treatments may stimulate germination and plant growth of many species (Ji et al., 2016; Ling et al., 2014; Meng et al., 2017; Stolárik et al., 2015; Tong et al., 2014) without provoking changes in the gene structure of the seeds as they are based on non-ionizing low level radiation (Jiafeng et al., 2014; Randeniya & de Groot, 2015; Zivkovic, Puac, Giba, Grubisic, & Petrovic, 2004). Since plasma treatments may both destroy pathogens and promote germination, the idea of using them for seed treatment prior to sowing appears to be realistic and promising. Cold plasma technology is considered suitable for seed treatment as it provides uniform treatments, does not destroy function of seeds, does not require chemicals and no environmental pollutants remain after the treatments. Additionally, treated seeds can again be stored if not used for germination (Dhayal, Lee, & Park, 2006).

The effect of non-thermal plasmas on seed-borne fungi that colonized the inside of seeds has not been studied extensively. Therefore, the objective of the present study was to investigate the effect of non-thermal plasmas with different gas compositions on the inactivation of D/P complex and the followed changes in seed quality as well as in antioxidant responses and in phytohormone balance. In particular, low-frequency (50 Hz) atmospheric pressure air DBD setups with two different dielectric barrier configurations operating at different discharge regimes and with oxygen and nitrogen as carrier gases were employed.

2. Materials and methods

2.1. Vegetal material

Soybean seeds corresponding to the variety DM 53i53 IPRO (GRUPO 5 CORTO INTACTA) were obtained from Don Mario Semillas S.A. and properly labeled and stored at 5 °C in the dark. Three replicates of one-hundred seeds from different seed lots were analyzed for D/P detection according to ISTA (2017) in order to evaluate the incidence of D/P complex and choose the lot with the highest incidence percentage to evaluate the efficiency of plasma treatments. Seeds with 16% of D/P incidence were finally employed. The infected seeds without treatment constituted the “infected control” (IC). Seeds completely free of D/P complex and without plasma treatment conformed the “healthy control” (HC).

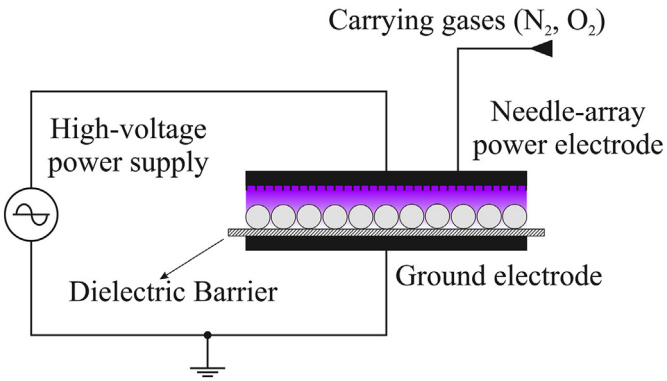

Fig. 1. Schematic diagram of the DBD plasma system for seed treatment.

2.2. DBD plasma source

A sketch of the DBD plasma source for the soybean seed treatment is shown in Fig. 1. The discharge consisted in a needle-array power electrode (with an overall diameter of 120 mm) and a plate ground electrode covered by a dielectric barrier of either 3 polyester films (400 μm thick Thernofase) or an arrangement of a thin phenolic sheet (2.5 mm in thickness) and 2 polyester films (100 μm thick Mylar). The gap between the upper surface of the barrier and the tip of the needles (tip radius 50 μm) was fixed to 10 mm during the experiment. The power supply was a high-voltage sine AC power supply (0–25 kV) operating at 50 Hz. Oxygen and nitrogen were injected into the discharge active region as carrier gases with a (measured) gas-flow rate of 6 NL min^{-1}. The electrical parameters of the discharge were monitored by using a 4-channel oscilloscope (Tektronix TDS 2004C with a sampling rate of 1 GS/s and an analogical bandwidth of 70 MHz). The discharge current was inferred from the measurement of the voltage drop across a shunt resistor (100 Ω) connected in series with the discharge, while the discharge voltage was measured by using a high-impedance voltage probe (Tektronix P6015A, 1000X, 3pf, 100 MΩ). The discharge input power (calculated from the measured current–voltage waveforms) was 65 W for the 1.2 mm-thick Thernofase barrier and 85 W for the 2.5 mm-thick Pertinax sheet with two Mylar films of 100 μm in thickness. The current and voltage waveforms of the DBD discharge are shown in Fig. 2 for a barrier of 2.5 mm-thick Pertinax sheet with two Mylar films of 100 μm in thickness, and nitrogen as carrier gas. The DBD current exhibits multiple pulses (peak current ~ 50 mA) of short duration in the positive half-cycle of the voltage oscillation (filamentary or streamer mode discharge), but a single wide pulse appears at the negative half-cycle (diffuse/homogenous mode discharge). On the contrary, the current waveform of the DBD with the polyester barrier (not shown) do not exhibits the diffuse mode at the negative voltage cycle. It is expected that the discharge mode of the DBD have influence on the production of the reactive species in the plasma (Brandenburg, 2017). The current pulses showed in Fig. 2 are related with the presence of thin plasma filaments bridging the electrode gas gap (filament radius ~ 50–100 μm) with current densities in the range 100 to 1000 A cm^{-2}, exhibiting a chaotic spatio-temporal behavior. The electron density n_e and temperature T_e are in the 10^{20}–10^{21} m^{-3} and 1–10 eV (1 eV = 11,600 K) ranges inside a plasma filament, while n_e and T_e are in the 10^{15}–10^{17} m^{-3} and 0.2–5 eV ranges

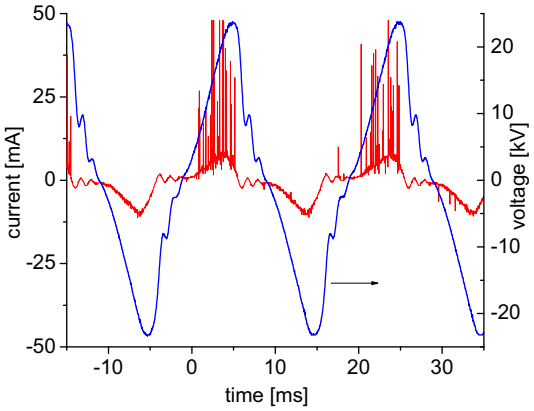

Fig. 2. Current and voltage waveforms of the DBD discharge for the Pertinax + Mylar barrier and nitrogen as carrier gas.

for a diffusive discharge. The short lifetime of the plasma filaments (1–10 ns) prevents the overheating of the discharge (< 10 °C in atmospheric pressure air), being the gas temperature T_g in the filament close to the background gas ~ 300 K (Brandenburg, 2017; Fridman, Chirokov, & Gutsol, 2005). This highly non-equilibrium state of the DBD discharge leads to the production of reactive species, ions, as well as additional electrons through electron-impact dissociation, excitation and ionization of background gas molecules. Of particular interest are reactive oxygen (ROS) and nitrogen (RNS) species that are abundantly present or can be easily produced from ambient air (Lu et al., 2016). Efforts to measure the concentration of ROS and RNS in the used DBD arrangements to correlate with the obtained results are currently in progress.

2.3. Plasma treatments

The plasma treatment of seeds was performed at an input power of 65 W for the 1.2 mm thick Thernofase barrier and 85 W for the 2.5 mm thick Pertinax sheet with 2 Mylar films of 100 μm in thickness. In each treatment, 500 soybean seeds were placed in the active plasma region on the dielectric barrier and the movement of the seeds was carried out mechanically to ensure uniform treatment. During the experiments the soybean seeds for each dielectric configuration and carrying gas were treated with plasma exposure times ranging from 60 to 180 s; as is shown in Table 1. The seeds were taken from the plasma discharge after treatment, and preserved in sterilized vessels before the biological experiments started.

Table 1
Description of non-thermal plasma treatments.

Dielectric barrier	Gas	Time exposure (minutes)	Nomenclature
Pertinax-Mylar	N_2	1	PMN1
		2	PMN2
		3	PMN3
	O_2	1	PMO1
		2	PMO2
		3	PMO3
Thernofase	N_2	1	TN1
		2	TN2
		3	TN3
	O_2	1	TO1
		2	TO2
		3	TO3
Infected control			IC
Healthy control			HC

2.4. Percentage of seeds infected by D/P complex

Three replicates of one-hundred seeds from each treatment were analyzed for D/P detection according to ISTA (2017). Seeds were placed on petri dishes with potato dextrose agar (PDA) and incubated under controlled conditions (25 ± 2 °C in the dark). After 7 days, the percentage of seeds infected with D/P complex (D/P %) was calculated.

$$\text{D/P (\%)} = (\text{Number of seeds infected with D/P complex /total number of seeds}) \times 100. \quad (1)$$

2.5. Germination

Four replicates of fifty seeds were analyzed according to the Top of Sand (TS) germination test for soybean proposed by ISTA (2014). The germination percentage reported indicates the proportion by number of seeds which have produced normal seedlings under 25 °C, light/dark (12 h/12 h) and within 7 days (ISTA, 2006).

$$\text{G (\%)} = (\text{Number of normal seedling/total number of seeds}) \times 100 \quad (2)$$

On a parallel test, four replicates of fifty seeds were guided in identical conditions. The germination percentage was recorded every 48 h for 7 days, germinated seeds were counted and radical protrusions of more than half of the seed length were registered as the criterion for germination sensu stricto. Total weight and length of seedlings were measured on day 7. Plant material for dry weight was dried at 80 °C for 120 h for measuring dry weight. Germination rate (GR) and Production rate (PR) were calculated according to Zhang et al. (2017). The germination index (GI) was calculated as described in the Association of Official Seed Analysts (AOSA, 1983). Vigor indexes I and II were calculated according to Abdul-Baki and Anderson (1973).

$$\text{GR (\%)} = (\text{Number of germinated seeds in 6 days/total number of seeds}) \times 100. \quad (3)$$

$$\text{PR (\%)} = \text{Seedling weight (g) on day 6} \times \text{GR (\%)/seed weight (g).} \quad (4)$$

$$\text{GI} = \sum (\text{Number of germinated seeds in t day/t day from sowing}). \quad (5)$$

$$\text{VI} = \text{Seedling length (cm)} \times \text{GR (\%)}/100. \quad (6)$$

$$\text{VII} = \text{Dry weight of seedling (g)} \times \text{GR (\%)}/100. \quad (7)$$

2.6. Seed imbibition

Dry seeds were weighed (m_0) and placed in plastic plates between moist papers during 24 h. Every 2 h, seeds were removed from the plates, dried with paper towel and weighed (m_t). The test was performed employing three replicates of 50 seeds. Percentage of water uptake was calculated for each time (t).

$$\text{Water uptake (\%)} = (m_t - m_0)/m_0 * 100 \quad (8)$$

Imbibition curves represent the time dependence of the average water absorption normalized by the initial masses of seeds.

2.7. Water droplet appearance

Distilled water droplets (5 μL) were placed on top of seeds and their appearances along 4 min were registered employing a microscopy camera (AxioCam ERc 5s Rev.2) coupled to a stereo-microscope (ZEISS, Stemi 508).

2.8. Lipid peroxidation

Seeds were placed between two layers of cotton imbibed with distilled water, at 25 °C. After 12 h, seedcoats were removed from seeds.

Imbibed seeds (1 g), seed coats (0.5 g) and dry seeds (1 g) were ground with 10 mL of 20% (w/v) trichloroacetic acid (TCA). Homogenates were centrifuged at 3000*g* for 20 min and the resulting supernatants were used for malondialdehyde (MDA) determination according to Heath and Packer (1968). The mixture for each assay contained 500 μL of the supernatant, 500 μL of 20% TCA with 0.5% (w/v) thiobarbituric acid (TBA) and 50 μL of 4% butylated hydroxytoluene (BHT) in ethanol. Mixtures were heated at 95 °C for 30 min, centrifuged at 3000*g* for 10 min. Finally, thiobarbituric acid-reactive substances (TBARS) were measured at 532 nm (A_1), and inespecific absorbances were determined at 600 nm (A_2). TBARS concentrations were obtained by subtracting the absorbances ($A_1 - A_2$) and employing the MDA extinction coefficient (155 mM^{-1} cm^{-1}). Results were expressed as nmol MDA g $tissue^{-1}$.

2.9. In situ localization of H_2O_2

Seeds were placed between two layers of cotton imbibed with distilled water for 6, 12 and 24 h, at 25 °C. After the corresponding time span of imbibition, imbibed seeds and dry seeds were incubated in a solution of 3,3′-diaminobenzidine stain in buffer Tris-HCl pH 6.5 (1 mg L^{-1}) at room temperature for 2 h. H_2O_2 was visualized as deposits of dark brown stain (Noriega, Santa Cruz, Batlle, Tomaro, & Balestrasse, 2012). Color intensity of deposits in each seed was measured employing Corel Photo-Paint X8 software. Variations on color intensity were calculated comparing the mean value of each group of seeds with the IC which was given arbitrary the value 100%.

2.10. Enzyme extraction

After 12 h of imbibition, 10 seeds (1.6 g) were ground and homogenized with 1 g polyvinylpyrrolidone (PVP) in 10 mL of 50 mM phosphate extraction buffer (pH 7.4) containing 1 mM EDTA and 0.5% (v/v) Triton X-100. The homogenates were centrifuged at 13,000*g* for 30 min at 4 °C and the supernatant fraction was used for the assays. Three biological replicates were performed.

2.11. Enzyme assays

Catalase activity (CAT) was measured at 25 °C according to Chance, Sies, and Boveris (1979). The enzyme assay contained 2 mM H_2O_2 in 50 mM phosphate buffer (pH 7.0) and 20 μL of enzyme extract in a total volume of 1 mL. CAT was estimated by the decrease in absorbance of H_2O_2 at 240 nm. Results were expressed in μmol min^{-1} mg $prot^{-1}$. Superoxide dismutase (SOD) activity was measured at 25 °C according to Becana, Aparicio-Tejo, Irigoyen, and Sanchez-Diaz (1986). The reaction mixture contained 2.2 μM riboflavin, 14.3 mM methionine, 82.5 μL nitro blue tetrazolium (NBT) in 50 mM phosphate buffer (pH 7.8), and 25–200 μL of enzyme extract in a final volume of 3 mL. SOD activity was assayed by measuring the ability of the enzyme extract to inhibit the photochemical reduction of NBT. Glass test tubes containing the mixture were shaken and placed 30 cm from a light bank of six 15 W fluorescent lamps. The reaction was allowed to run for 10 min and stopped by switching the light off. The reduction in NBT was followed by reading absorbance at 560 nm every 2 min. Identical tubes without enzyme extract were used as blanks. One unit of SOD was defined as the enzyme activity which inhibited the photo-reduction of NBT to blue formazan by 50%, and SOD activity of the extracts was expressed as U mg $protein^{-1}$. Protein content of the extracts was determined according to the method of Bradford (1976).

2.12. Reduced glutathione (GSH) determination

Non-protein thiols were extracted from seeds after 12 h of imbibition, employing HCl 0.1 N (pH 2.0) and a dilution ratio of 1:10 w/v (Schupp & Rennenberg, 1988). GSH was oxidized by DTNB (5,5-dithiobis-(2-nitrobenzoic acid)) resulting in the formation of GSSG (glutathione disulfide) and TNB (5-thio-2-nitrobenzoic acid). GSSG was then reduced to GSH by GR (glutathione reductase) using reducing equivalent provided by NADPH (nicotinamide adenine dinucleotide phosphate). The rate of TNB formation is proportional to the sum of GSH and GSSG (i.e. total glutathione present in the sample) and was determined by measuring the formation of TNB at 412 nm. GSSG was determined using 2-vinylpyridine. Sample concentrations of total glutathione and GSSG were determined by experimentally derived standard curves. GSH was calculated as the difference between total glutathione and GSSG (Anderson, 1985; Griffith, 1980) and expressed as nmol g dw^{-1}.

2.13. Phytohormones

Seeds were placed between two layers of cotton imbibed with distilled water, at 25 °C for 12 h. Imbibed seeds were cut into small segments, homogenized, and 50 mg of tissues were used in four repetitions for each treatment. Phytohormones extraction, purification and quantification were performed according to Dobrev and Kamınek (2002) with modifications. Abscisic acid (ABA) and indole acetic acid (IAA) contents were analyzed by HPLC (Agilent 1100 Series). Ethylene production was measured according to Ishibashi, Koda, Zheng, Yuasa, and Iwaya-Inoue (2012). Forty seeds were placed in a 120 mL flask between two layers of cotton imbibed with distilled water. Each flask was then sealed with cling film, and a 1.0-mL gas sample after imbibition for 24 and 48 h was removed using a syringe. The samples were examined on a gas chromatograph (Khon 3000 HRGC) and ethylene was quantified by comparison of peak areas with those produced by known amounts of the hormone. Ethylene production was normalized dividing the content by the number of seeds in each flask.

2.14. Statistical analysis

All data presented correspond to the mean value ± standard error (SE) of three replicates. Analyses were performed using the statistic software package RCommander version 3.1.2 (2014). After testing for the assumption of the normal distribution, the variance ($P < 0.05$) of the obtained data was analyzed by one-way analysis of variance (ANOVA). Dunnett's tests were used for many-to-one comparisons and Honestly-significant-difference (Tukey HSD) was obtained for all pairwise comparisons, at $P < 0.05$. The results were showed as mean value ± standard error (SE) of three replicates in each group.

3. Results and discussion

3.1. Seed quality

3.1.1. Seed health

In the discharge plasma process the formation of plasma components (electrons, UV and active species) depends on the discharge plasma gas sources (Meng et al., 2017) and the duration of exposure (Li et al., 2017; Tong et al., 2014), among several others factors. It is well documented that different discharge plasmas affect treatment efficacy when used either for enhancing seed germination and plant growth (Kitazaki, Sarinont, Koga, Hayashi, & Shiratani, 2014; Ling et al., 2014; Meng et al., 2017; Mitra et al., 2014; Tong et al., 2014) or for controlling food surface microorganisms (Dasan et al., 2016; Laroussi & Leipold, 2004; Mitra et al., 2014) as there is an optimum beneficial radical dose. In this context, finding the optimum combination of gas source and time of exposure for controlling seed-borne pathogens without provoking damage to the seed, constituted the point of departure of the present investigation. Twelve plasma treatments were analyzed for seed health in order to select the ones with better performance in decreasing the number of seeds infected by D/P complex. Previous experiments (data not shown) led us to design these treatments based on the combination of two different barriers (Thernofase

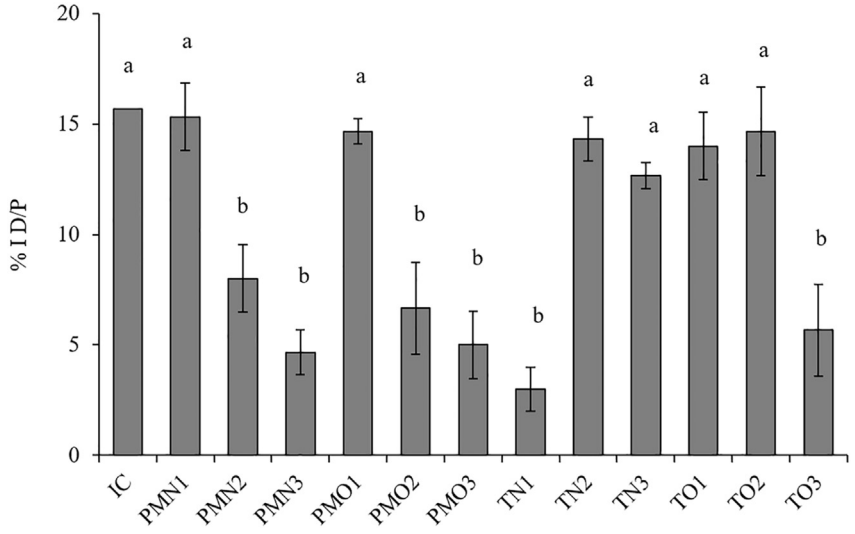

Fig. 3. Effect of cold plasma on the percentage of seeds infected by D/P complex. Error bars indicate standard error ($n = 3$). Different lowercase letters denote statistical differences between treatment groups and IC (Dunnett's test, $P < 0.05$).

and Pertinax + Mylar), two gases (O_2 and N_2) and three times of exposure (one, two and 3 min). Significant differences were detected between IC and treatments PMN2, PMN3, PMO2, PMO3, TN1 and TO3 (Fig. 3), with reductions of about 49–81% of the initial percentage of seeds infected by D/P complex.

Our results are consistent with Sélcuk, Oksuz, and Basaran (2008), Dasan et al. (2016), Iseki et al. (2010), Ishikawa et al. (2012) and Hashizume et al. (2013) who found that cold plasma was effective inactivating contaminant fungi (*Aspergillus* spp. and *Penicillium* spp.) on the surface of seeds without affecting seed germination. But our study is the first to address the control of pathogenic fungi that colonize the inside of seeds and it is also the first to report the control of soybean seed-borne pathogens employing cold plasma. D/P complex is known to provoke severe damage to soybean seed quality and to be capable of invading and rapidly rotting seed during the early stages of germination (Kmetz, Schmitthenner, & Ellett, 1978). As seed deterioration is characterized by physiological manifestations such as increased electrolyte leakage, reduced germinability and increased number of abnormal seedlings (Abdul-Baki & Anderson, 1972), the next step was to analyze how these parameters were being affected by plasma exposure.

3.1.2. Germination and vigor

Considering the recovery of seed health evidenced in the previous analysis, four of the best treatments (PMN3, PMO2, TN1 and TO3) were selected to continue investigating plasma effects on the quality of seeds by testing germinability and vigor (Fig. 4).

All plasma treatments had a significant stimulatory effect on seed germination and vigor indicating that, if the appropriate dose of plasma is used, seeds germinate better and faster and produce longer and heavier seedlings.

These results were in accordance with several researches carried out on seed priming and enhancing germination effects of cold plasma (Bormashenko, Grynyov, Bormashenko, & Drori, 2012; Dobrin, Magureanu, Mandache, & Ionita, 2015; Ji et al., 2016; Li et al., 2017; Ling et al., 2014; Meng et al., 2017; Stolárik et al., 2015; Tong et al., 2014).

3.1.3. Water uptake

Differences between treatments and controls were noticeable throughout entire time span of the experiment (Fig. 5). IC showed the highest levels of water uptake, an expected behavior considering that seeds were highly infected by D/P fungi, a group of pathogens well known to remove protective value of seed coat tissues resulting in general loss of seed quality (Li, 2011; Rupe & Luttrell, 2008). This observation was supported by the values of seed quality showed above, where the evident loss in germination of IC group responded mostly to seed rot or production of abnormal seedling. On the other hand, treated seeds curves showed stimulated water absorption compared with HC. These results were in accordance with other studies (Dobrin et al., 2015; Ji et al., 2016; Li et al., 2017; Ling et al., 2014; Meng et al., 2017; Stolárik et al., 2015) and suggested that plasma treatment provoked changes on seed permeability to water, an effect positively correlated with seed quality improvement showed before.

3.1.4. Appearance of water droplet and lipid peroxidation of seed coats

Many authors (Bormashenko et al., 2012; Ji et al., 2016; Li et al., 2017; Stolárik et al., 2015) observed that moderate erosion or oxidation of the seed coat by plasma benefited seed germination as altered seed coat increased its hydrophilic ability and Ling et al. (2014) proposed that this variation in seed hydrophilicity could be verified by modifications in the apparent contact angle between water droplet and seed coat (greater for treated seeds). Therefore we decided to evaluate the behavior of water droplet in contact with seed surfaces and captured in Fig. 6A, where differences in water droplet form between treated and not treated seeds may be easily observed as well as the differential speed at which the drop is absorbed by the coat. According to Dann (1970) flatter water droplets mean that polar and nonpolar surface energy (hydrophilicity) increased. With the aim of clarifying the causes of the observed modifications on seed coats hydrophilicity, these results were accompanied by the analysis of lipid peroxidation of seed coats. As Fig. 6B shows, the lipid peroxidation of seed coats of treated seeds increased between 21 and 90% compared with IC and were 54–143% higher than HC. Lipid peroxidation of IC seed coats was 28% greater than HC, as expected considering the high presence of D/P fungi in the first group. Our results indicated a strong and positive correlation between seed hydrophilicity and lipid peroxidation of seed coats for all the treatments. Considering that changes in the structural properties of membrane bilayer by oxidative damage are known to increased membrane permeability to water (Wong-Ekkabut et al., 2007), our findings indicate that plasma-produced ROS provoked the oxidation of lipids present in seed coats triggering the observed changes in water properties and the subsequent chain of effects that finally led to germination enhancement. On the other hands, in our study no recovery of hydrophobicity was observed even after 4 months from treatment (data not showed). This singularity was also observed by Bormashenko et al. (2012) in lentils, beans and wheat.

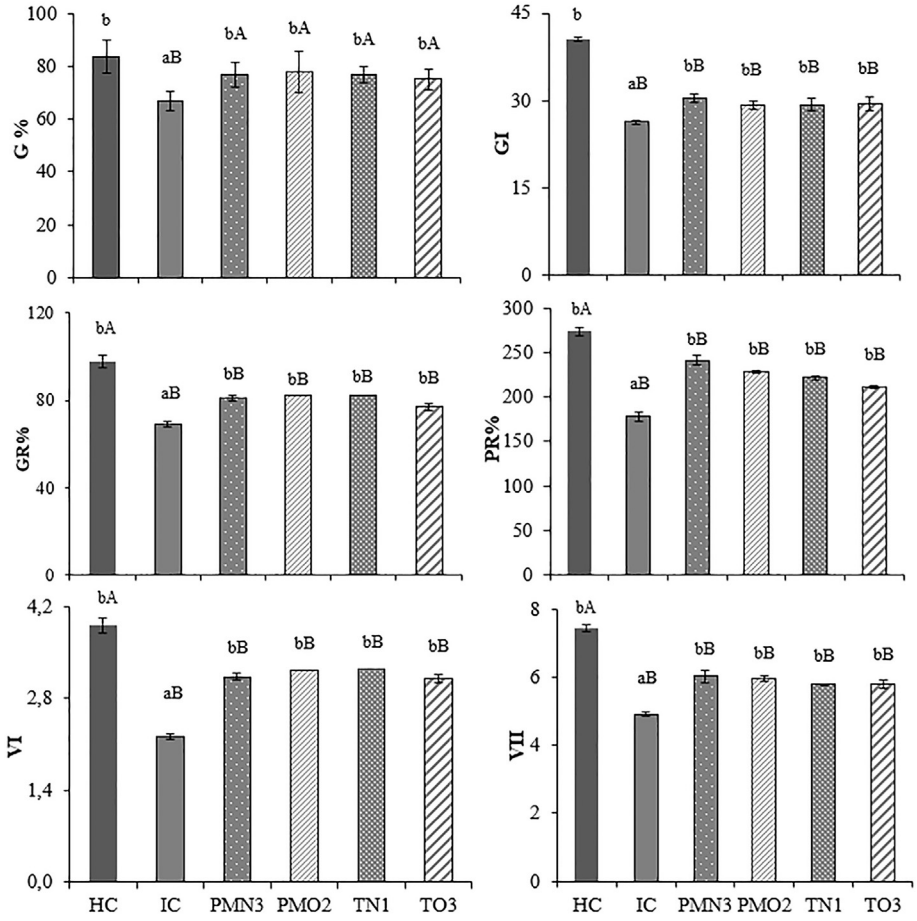

Fig. 4. Effect of cold plasma on germination (G%), germination rate (GR%), germination index (GI), production rate (PR%) and vigor (VI and VII). Error bars indicate standard error ($n = 3$). Different lowercase letters denote statistical differences between treatment groups and positive control; different capital letters denote statistical differences between treatment groups and negative control (Dunnett's test, $P < 0.05$).

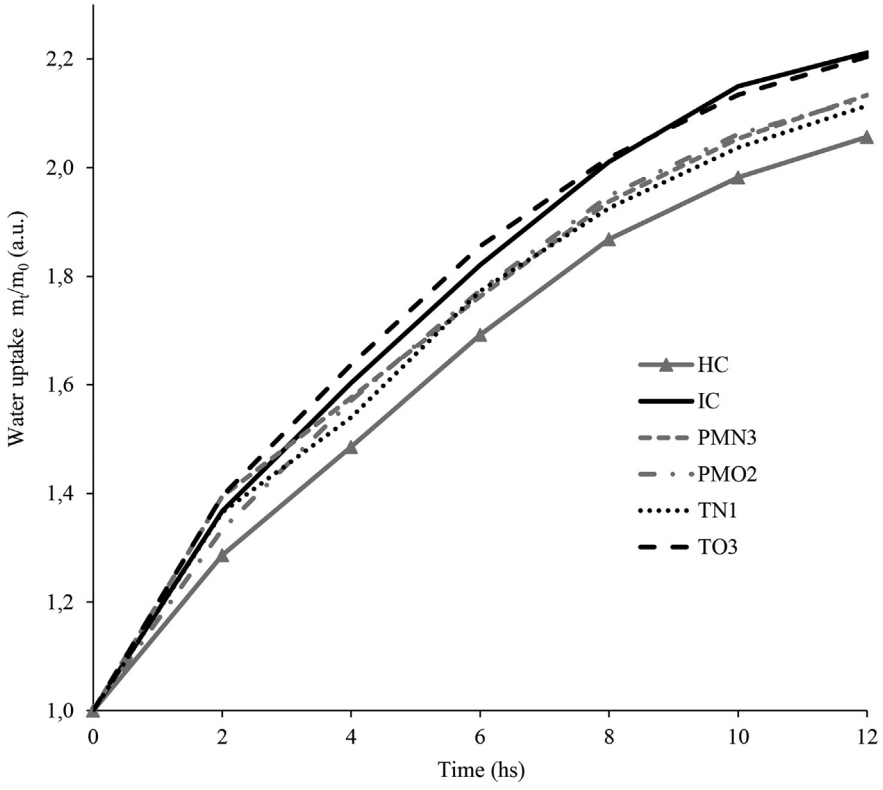

Fig. 5. Effect of cold plasma on the water uptake of soybean seeds from 0 to 12 h of imbibition.

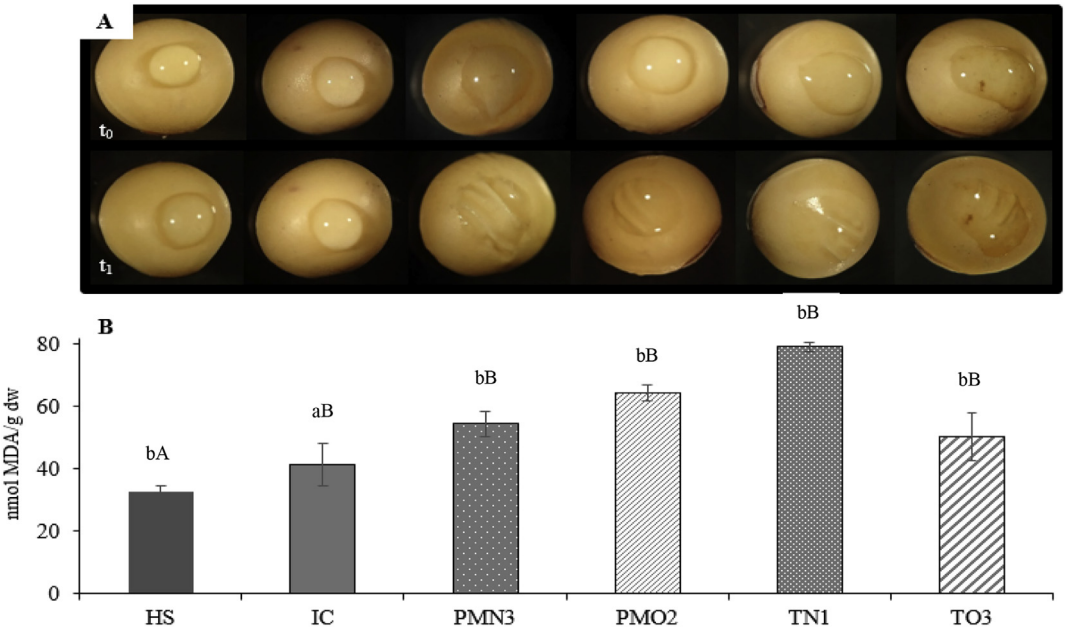

Fig. 6. A) Appearance of water droplets placed on seeds: t_0 initial time, t_1 4 min later. B) TBARS of seed coats after 12 h of imbibition. Error bars indicate standard error ($n = 3$). Different lowercase letters denote statistical differences between treatment groups and positive control; different capital letters denote statistical differences between treatment groups and negative control (Dunnett's test, $P < 0.05$).

3.2. Physiological responses

3.2.1. Oxidative stress

It has been well documented that environmental, chemical, physical and biotic stresses disrupt the metabolic balance of cells resulting in enhanced production of ROS; plants have evolved protective mechanisms against these stresses that allow them to activate or regulate their enzymatic and non-enzymatic antioxidant defenses in order to maintain a low steady-state level of ROS in cells and thus cope with oxidative damage (Apel & Hirt, 2004; Balestrasse, Gardey, Gallego, & Tomaro, 2001; Bustingorri, Balestrasse, & Lavado, 2015; Mittler, Vanderauwera, Gollery, & Van Breusegem, 2004; Santa-Cruz et al., 2017). Plasma treatment itself is supposed to be stressful for the seed as it is comprised of several excited reactive species, electrons, positive and negative ions, free radicals, gas molecules and quanta of electromagnetic radiation (Misra et al., 2016). In order to evaluate the physiological responses of seeds to plasma treatment, oxidative stress parameters and phytohormones were determined.

Table 2 shows the effects of cold plasma on lipid peroxidation of dry and imbibed seeds. No differences were detected in MDA content between treated seeds and HC, neither for dry nor for imbibed seeds. These results indicated that plasma treatment did not provoke lipid peroxidation in exposed seeds. IC exhibited the greatest lipid peroxidation in both conditions (dry and imbibed), as expected considering that seeds were highly infected by D/P fungi. Lipid peroxidation results were accompanied by in situ localization of H_2O_2 along 24 h of imbibition (Fig. 7) and by measures of changes in color intensity of the corresponding stains in comparison with IC (color intensity = 100%) at different times from the beginning of imbibition (Table 3).

Table 2
Effect of cold plasma on lipid peroxidation of dry and imbibed seeds.

Treatment	Thiobarbituric acid reactive substances (nmol malondialdehyde g tissue^{-1})							
	Dry seed				Imbibed seed			
HC	30,59	± 2,28	b	A	14,93	± 1,98	a	A
IC	39,09	± 0,51	a	B	15,52	± 0,48	a	A
PMN3	29,50	± 3,75	b	A	12,31	± 1,14	a	A
PMO2	28,79	± 0,65	b	A	14,65	± 1,49	a	A
TN1	28,78	± 4,20	b	A	13,24	± 1,25	a	A
TO3	28,05	± 3,36	b	A	13,25	± 1,24	a	A

Different lowercase letters denote statistical differences between treatment groups and IC; different capital letters denote statistical differences between treatment groups and HC (Dunnett's test, $P < 0.05$).

According to Puntarulo, Galleano, Sanchez, and Boveris (1991), the presence of H_2O_2 in germinating soybean seeds achieves the highest level at 6 h from the beginning of imbibition; this level decreases until hour twelve and then increases again along hours until hour twenty-four. In our study, positive values of variation on color intensity at 6 h indicated lower content of H_2O_2 in the IC than in treated seeds and the HC. Moreover, at 12 h, treated seeds presented significant reductions in peroxide content compared with IC (which explain the negative values of variation on color intensity). Our results coincided with Puntarulo et al. (1991), demonstrating that treated seeds and the HC were germinating actively and that the high presence of D/P fungi in IC clearly delayed the natural stages of germination process propitiating fungal colonization and ending out, most of the times, in seed rot. These results agree with the seed health and quality (Figs. 3 and 4).

As exposed in Fig. 8, treated seeds presented values of CAT activity lower than IC and similar to HC. SOD activity showed no detectable differences between treated seeds and controls; TN1 showed the highest SOD activity but differed significantly only from HC. For GSH, results showed lower GSH content for treatments and HC in comparison to IC. Low CAT activity together with low GSH content and low lipid peroxidation exhibited by treated seeds suggested that the H_2O_2 generated during exposure to plasma did not activate antioxidant defenses neither provoke damage to cell membrane. Nevertheless, the produced H_2O_2 could have been acting as a signal molecule in the induction of germination (Puntarulo et al., 1991; Wojtyla, Lechowska, Kubala, & Garnczarska, 2016). High CAT activity and GSH content exhibited by infected seeds (IC) suggested that they coped with the oxidative damage caused by biotic stress by increasing enzymatic and non-enzymatic scavenging of H_2O_2. The antioxidant responds and peroxide production were detected by the first time in soybean seed in relation to the presence of D/P complex.

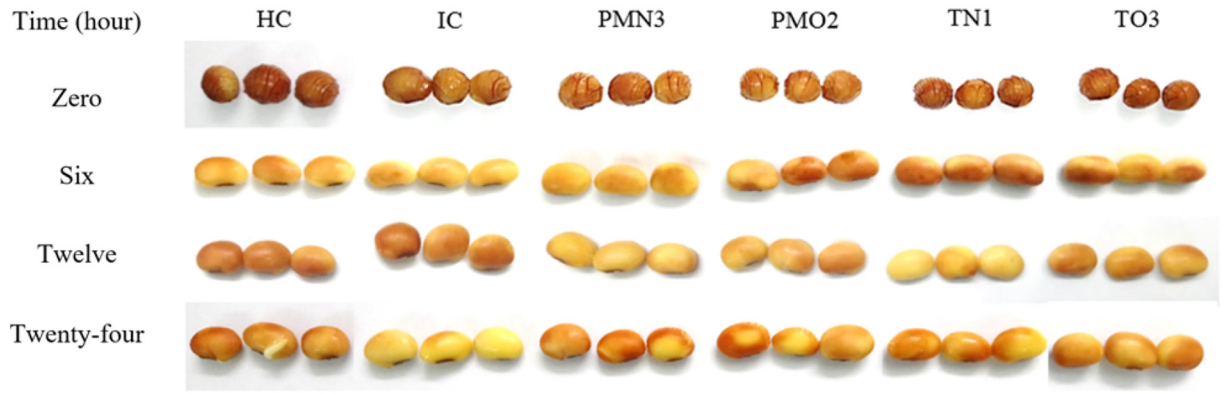

Fig. 7. In situ localization of H_2O_2 along 24 h of seed imbibition.

Table 3
Percentage of variation on the color intensity of stain deposits of H_2O_2 on seeds in comparison with IC at different times of imbibition.

Time of imbibition (hour)	Variation on color intensity (%)				
	HC	PMN3	PMO2	TN1	TO3
Zero	32	6	3	26	28
Six	24	19	32	49	45
Twelve	-2	−40	−31	−42	−20
Twenty-four	70	74	53	62	67

IC color intensity = 100%.

3.2.2. Phytohormones

ABA and AIA contents of imbibed seeds are showed on Fig. 9A. ABA content declined in every treatment exhibiting values from 14 to 37% lower than IC. Considering the fact that seed ABA content is defined before harvesting and that HC seeds were produced under different field conditions and harvested earlier than the other groups, their differences in respect to ABA content should not be considered. Our results demonstrated that plasma treatment inhibited the synthesis of ABA in seeds. Even more, ABA content and germination exhibited a negative correlation. A similar pattern was observed by Quebedeaux, Sweetser, and Rowell (1976), Fountain and Bewley (1976), Ackerson (1984) and Brenner and Cheikh (1995), who proposed that ABA played a regulatory role in developing soybean, suppressing germination; along seed maturation, ABA content declined gradually to a minimal level that allowed seed to germinate when matured. Recently, Shu et al. (2017) found that ABA content correlated negatively with soybean germination under abiotic stress, confirming the participation of this phytohormone in the regulation of soybean seed germination. On the other hand, IAA content was stimulated by plasma treatment, correlating positively with germination parameters (Fig. 4). According to Sauer, Robert, and Kleine-Vehn (2013), IAA is able to affect seed germination by regulating the activity of certain enzymes, resulting in higher rates of cell growth and development. When evaluating seed ethylene content (Fig. 9B) we observed stimulated production of this phytohormone after 24 h of imbibition. This result coincided with an increase of H_2O_2 showed in Fig. 7. Our findings agreed with Ishibashi, Tawaratsumida, Zheng, Yuasa, and Iwaya-Inoue (2010) and Ishibashi et al. (2012) who demonstrated that H_2O_2 was a signal molecule in ethylene production and that exogenous supplies of H_2O_2 stimulated root tip cell growth by increasing ethylene production. On the other hand, it has been well documented that ethylene levels inside plant tissues increase following pathogen infection (Johnson & Ecker, 1998; Van Loon, Geraats, & Linthorst, 2006) and that ethylene emitted by infected seed facilitates fungal colonization (Wang et al., 2017). The plasma treatments (PMN3, TN1 and TO3) reduced ethylene levels (Fig. 9B). Therefore our result demonstrated that H_2O_2 content (Fig. 7)

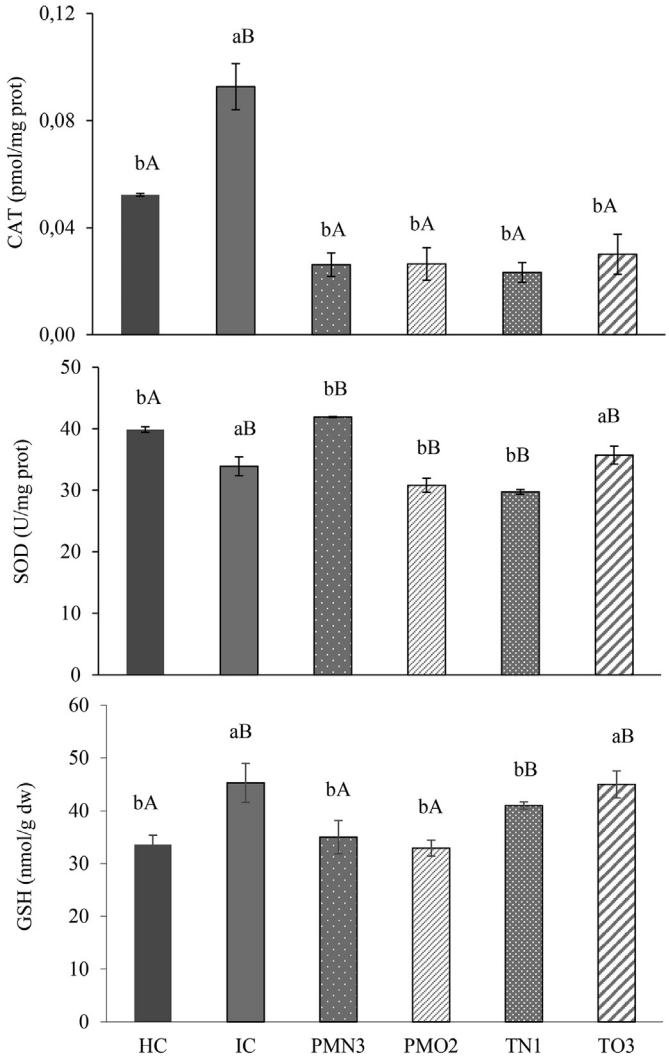

Fig. 8. Effect of cold plasma on CAT activity, SOD activity and GSH content after 12 h of imbibition. Error bars indicate standard error ($n = 3$). Different lowercase letters denote statistical differences between treatment groups and IC; different capital letters denote statistical differences between treatment groups and HC (Dunnett's test, $P < 0.05$).

correlated negatively with ABA content and positively with AIA content, ethylene production (Fig. 9A and B) and germination parameters (Fig. 4). These findings suggested that H_2O_2 was involved in the regulation of phytohormones content and, thereby in the improvement of seed germination. In accordance with this, Barba-Espín et al. (2010,

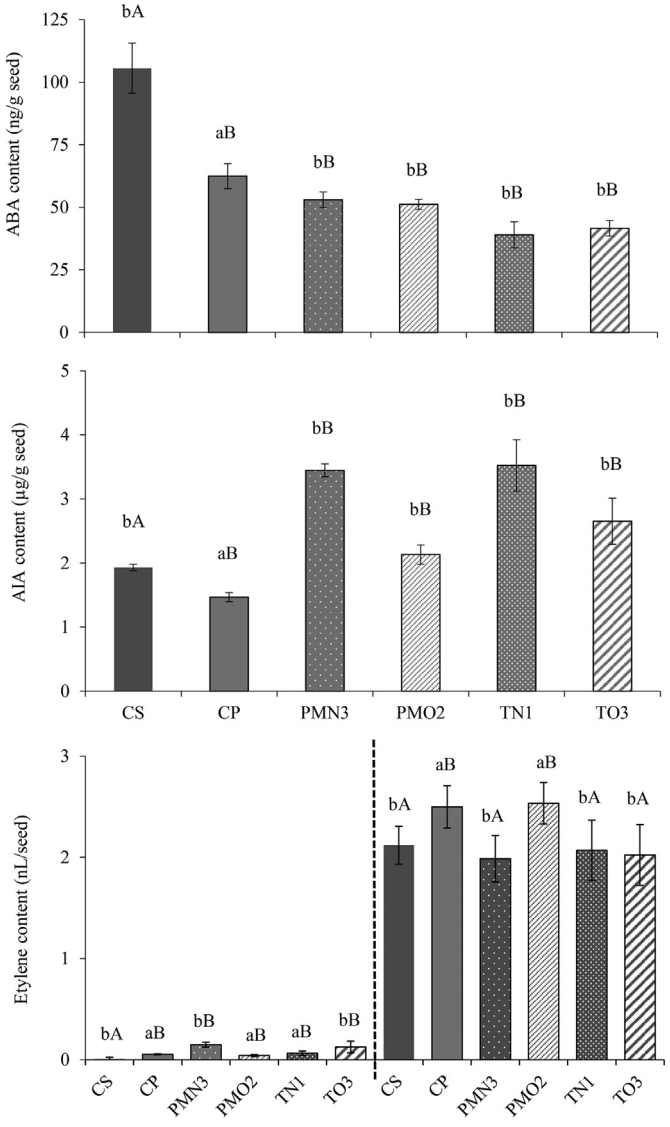

Fig. 9. A) Effect of cold plasma on ABA and AIA content after 12 h of imbibition. B) Effect of cold plasma on Ethylene content at 24 (to the left of the dashed line) and 48 h (to the right of the dashed line) from the beginning of imbibition. Error bars indicate standard error ($n = 3$). Different lowercase letters denote statistical differences between treatment groups and IC; different capital letters denote statistical differences between treatment groups and HC (Dunnett's test, $P < 0.05$).

2011) proposed that H_2O_2 could act as a signal molecule in the regulation of catabolism and biosynthesis of various phytohormones involved in germination and seedling growth.

4. Conclusions

Our study is the first to address the control of pathogenic fungi that colonize the inside of soybean seeds employing cold plasma. This work was conducted employing seed samples that came from infected and non-infected fields, leaving aside the artificial inoculation. The opportunity of working with a real field situation provided great impact to our investigation.

We used two arrangements of atmospheric pressure air DBD plasma generators operating at 50 Hz by using two different barrier types to study the effects of cold plasma on seed-borne pathogens (D/P complex) and on germination parameters of soybean seed. No significant differences were detected in the effects of each treatment along the study; in spite of the differences in the discharge mode of the tested DBD setups.

We concluded that plasma treatment: improved seed health (by reducing the percentage of seeds infected with D/P fungi), and provoked changes in seed antioxidant profile and in phytohormone balance (all orchested by H_2O_2), without causing oxidative damage to seed cell membranes. On the other hand, we observed that plasma-produced ROS provoked the oxidation of lipids present in seed coats leading to promoted seed water uptake (by increasing seed coats hydrophilicity). All mentioned above contributed to the enhancement of soybean seed germination and vigor.

Acknowledgements

This work was supported by grants from CONICET (PIP 11220120100453), Universidad Tecnológica Nacional (PID 4626), Agencia Nacional de Promoción Científica y Tecnológica (PICT 2015 N°1553) and Universidad de Buenos Aires (UBACYT 20020120100145). K. B., C. Z. and L. P. are members of CONICET. E. C. thanks CONICET for his doctoral fellowship. M.C.P.P. thanks Agencia Nacional de Promoción Científica y Tecnológica for her doctoral fellowship.

We thank Patricia del Fueyo (Seed Laboratory - Universidad de Buenos Aires, Facultad de Agronomía, Argentine) and Pablo Grijalba (Phytopathology - Universidad de Buenos Aires, Facultad de Agronomía, Argentine) who provided technical support in seed quality evaluation techniques that greatly assisted the research.

References

Abdul-Baki, A. A., & Anderson, J. D. (1973). Vigour determination in soybean seed by multiplication. *Crop Science, 3*, 630–633.
Abdul-Baki, A. A., & Anderson, J. D. (1972). Physiological and biochemical deterioration of seeds. *Seed Biology, 2*, 283–316.
Ackerson, R. C. (1984). Abscisic acid and precocious germination in soybeans. *Journal of Experimental Botany, 35*(3), 414–421.
Anderson, M. E. (1985). Determination of glutathione and glutathione disulfide in biological samples. *Methods in enzymology. Vol. 113. Methods in enzymology* (pp. 548–555). Academic Press.
AOSA (1983). *Seed vigor testing handbook. Contribution No. 32 to the handbook on Seed Testing*Association of Official Seed Analysis.
Apel, K., & Hirt, H. (2004). Reactive oxygen species: Metabolism, oxidative stress, and signal transduction. *Annual Review of Plant Biology, 55*, 373–399.
Balestrasse, K. B., Gardey, L., Gallego, S. M., & Tomaro, M. L. (2001). Response of antioxidant defense system in soybean nodules and roots subjected to cadmium stress. *Functional Plant Biology, 28*(6), 497–504.
Barba-Espín, G., Diaz Vivancos, P., Clemente Moreno, M. J., Albacete, A., Faize, L., Faize, M., & Hernández, J. A. (2010). Interaction between hydrogen peroxide and plant hormones during germination and the early growth of pea seedlings. *Plant, Cell & Environment, 33*(6), 981–994.
Barba-Espín, G., Diaz Vivancos, P., Job, D., Belghazi, M., Job, C., & Hernández, J. A. (2011). Understanding the role of H2O2 during pea seed germination: A combined proteomic and hormone profiling approach. *Plant, Cell & Environment, 34*(11), 1907–1919.
Basaran, P., Basaran-Akgul, N., & Oksuz, L. (2008). Elimination of *Aspergillus parasiticus* from nut surface with low-pressure cold plasma (LPCP) treatment. *Food Microbiology, 25*(4), 626–632.
Becana, M., Aparicio-Tejo, P., Irigoyen, J. J., & Sanchez-Diaz, M. (1986). Some enzymes of hydrogen peroxide metabolism in leaves and root nodules of *Medicago sativa*. *Plant Physiology, 82*(4), 1169–1171.
Bormashenko, E., Grynyov, R., Bormashenko, Y., & Drori, E. (2012). Cold radiofrequency plasma treatment modifies wettability and germination speed of plant seeds. *Scientific Reports, 2*, 741.
Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry, 72*(1–2), 248–254.
Brandenburg, R. (2017). Dielectric barrier discharges: Progress on plasma sources and on the understanding of regimes and single filaments. *Plasma Sources Science and Technology, 26*(5), 053001.
Brenner, M. L., & Cheikh, N. (1995). The role of hormones in photosynthate partitioning and seed filling. *Plant hormones* (pp. 649–670). Springer.
Bustingorri, C., Balestrasse, K., & Lavado, R. S. (2015). Efecto de altas concentraciones de arsénico y flúor en el suelo sobre plantas de soja. *Phyton (Buenos Aires), 84*(2), 407–416.
Carvalho, F. P. (2006). Agriculture, pesticides, food security and food safety. *Environmental Science & Policy, 9*(7–8), 685–692.
Chance, B., Sies, H., & Boveris, A. (1979). Hydroperoxide metabolism in mammalian organs. *Physiological Reviews, 59*(3), 527–605.
Daeschlein, G., Napp, M., Von Podewils, S., Lutze, S., Emmert, S., Lange, A., & Jünger, M. (2014). In vitro susceptibility of multidrug resistant skin and wound pathogens

against low temperature atmospheric pressure plasma jet (APPJ) and dielectric barrier discharge plasma (DBD). *Plasma Processes and Polymers, 11*(2), 175–183.
Dann, J. R. (1970). Forces involved in the adhesive process: I. Critical surface tensions of polymeric solids as determined with polar liquids. *Journal of Colloid and Interface Science, 32*(2), 302–320.
Dasan, B. G., Mutlu, M., & Boyaci, I. H. (2016). Decontamination of *Aspergillus flavus* and *Aspergillus parasiticus* spores on hazelnuts via atmospheric pressure fluidized bed plasma reactor. *International Journal of Food Microbiology, 216*, 50–59.
Dhayal, M., Lee, S. Y., & Park, S. U. (2006). Using low-pressure plasma for *Carthamus tinctorium* L. seed surface modification. *Vacuum, 80*(5), 499–506.
Dobrev, P. I., & Kamınek, M. (2002). Fast and efficient separation of cytokinins from auxin and abscisic acid and their purification using mixed-mode solid-phase extraction. *Journal of Chromatography A, 950*(1–2), 21–29.
Dobrin, D., Magureanu, M., Mandache, N. B., & Ionita, M. D. (2015). The effect of non-thermal plasma treatment on wheat germination and early growth. *Innovative Food Science & Emerging Technologies, 29*, 255–260.
Fountain, D. W., & Bewley, J. D. (1976). Lettuce seed germination: Modulation of pregermination protein synthesis by gibberellic acid, abscisic acid, and cytokinin. *Plant Physiology, 58*(4), 530–536.
Fridman, A., Chirokov, A., & Gutsol, A. (2005). Non-thermal atmospheric pressure discharges. *Journal of Physics D: Applied Physics, 38*, R1–R24.
Fridman, G., Friedman, G., Gutsol, A., Shekhter, A. B., Vasilets, V. N., & Fridman, A. (2008). Applied plasma medicine. *Plasma Processes and Polymers, 5*(6), 503–533.
Griffith, O. W. (1980). Determination of glutathione and glutathione disulfide using glutathione reductase and 2-vinylpyridine. *Analytical Biochemistry, 106*, 207–212.
Hahn, M. (2014). The rising threat of fungicide resistance in plant pathogenic fungi: *Botrytis* as a case study. *Journal of Chemical Biology, 7*(4), 133–141.
Hashizume, H., Ohta, T., Fengdong, J., Takeda, K., Ishikawa, K., Hori, M., & Ito, M. (2013). Inactivation effects of neutral reactive-oxygen species on *Penicillium digitatum* spores using non-equilibrium atmospheric-pressure oxygen radical source. *Applied Physics Letters, 103*(15), 153708.
Heath, R. L., & Packer, L. (1968). Photoperoxidation in isolated chloroplasts: I. Kinetics and stoichiometry of fatty acid peroxidation. *Archives of Biochemistry and Biophysics, 125*(1), 189–198.
Iseki, S., Ohta, T., Aomatsu, A., Ito, M., Kano, H., Higashijima, Y., & Hori, M. (2010). Rapid inactivation of *Penicillium digitatum* spores using high-density nonequilibrium atmospheric pressure plasma. *Applied Physics Letters, 96*(15), 153704.
Ishibashi, Y., Koda, Y., Zheng, S. H., Yuasa, T., & Iwaya-Inoue, M. (2012). Regulation of soybean seed germination through ethylene production in response to reactive oxygen species. *Annals of Botany, 111*(1), 95–102.
Ishibashi, Y., Tawaratsumida, T., Zheng, S. H., Yuasa, T., & Iwaya-Inoue, M. (2010). NADPH oxidases act as key enzyme on germination and seedling growth in barley (*Hordeum vulgare* L.). *Plant Production Science, 13*(1), 45–52.
Ishikawa, K., Mizuno, H., Tanaka, H., Tamiya, K., Hashizume, H., Ohta, T., & Sekine, M. (2012). Real-time in situ electron spin resonance measurements on fungal spores of *Penicillium digitatum* during exposure of oxygen plasmas. *Applied Physics Letters, 101*(1), 013704.
ISTA (2006). *Handbook on seedling evaluation* (3rd edition). Switzerland: International Seed Testing Association.
ISTA (2014). *The germination test. Vol. 2014*International Seed Testing Association (Chapter 5).
ISTA (2017). *Validated seed health testing methods. Vol. 2017*International Seed Testing Association (Chapter 7 (7-016)).
Ji, S. H., Choi, K. H., Pengkit, A., Im, J. S., Kim, J. S., Kim, Y. H., & Park, G. (2016). Effects of high voltage nanosecond pulsed plasma and micro DBD plasma on seed germination, growth development and physiological activities in spinach. *Archives of Biochemistry and Biophysics, 605*, 117–128.
Jiafeng, J., Xin, H., Ling, L. I., Jiangang, L., Hanliang, S., Qilai, X., & Yuanhua, D. (2014). Effect of cold plasma treatment on seed germination and growth of wheat. *Plasma Science and Technology, 16*(1), 54.
Johnson, P. R., & Ecker, J. R. (1998). The ethylene gas signal transduction pathway: a molecular perspective. *Annual Review of Genetics, 32*(1), 227–254.
Kitazaki, S., Sarinont, T., Koga, K., Hayashi, N., & Shiratani, M. (2014). Plasma induced long-term growth enhancement of *Raphanus sativus* L. using combinatorial atmospheric air dielectric barrier discharge plasmas. *Current Applied Physics, 14*, 149–153.
Kmetz, K. T., Schmitthenner, A. F., & Ellett, C. W. (1978). Soybean seed decay: Prevalence of infection and symptom expression caused by *Phomopsis* sp., *Diaporthe phaseolorum* var. *sojae*, and *D. phaseolorum* var. *caulivora*. *Phytopathology, 68*(6), 836–841.
Laroussi, M. (2005). Low temperature plasma-based sterilization: Overview and state of the art. *Plasma Processes and Polymers, 2*(5), 391–400.
Laroussi, M., & Leipold, F. (2004). Evaluation of the roles of reactive species, heat, and UV radiation in the inactivation of bacterial cells by air plasmas at atmospheric pressure. *International Journal of Mass Spectrometry, 233*(1–3), 81–86.
Li, S. (2011). Phomopsis seed decay of soybean. *Soybean-molecular aspects of breeding* (pp. 277–293). InTech.
Li, Y., Wang, T., Meng, Y., Qu, G., Sun, Q., Liang, D., & Hu, S. (2017). Air atmospheric dielectric barrier discharge plasma induced germination and growth enhancement of wheat seed. *Plasma Chemistry and Plasma Processing, 37*(6), 1621–1634.
Ling, L., Jiafeng, J., Jiangang, L., Minchong, S., Xin, H., Hanliang, S., & Yuanhua, D. (2014). Effects of cold plasma treatment on seed germination and seedling growth of soybean. *Scientific Reports, 4*, 5859.
Lu, X., Naidis, G. V., Laroussi, M., Reuter, S., Graves, D. B., & Ostrikov, K. (2016). Reactive species in non-equilibrium atmospheric-pressure plasmas: Generation, transport, and biological effects. *Physics Reports, 630*, 1–84.
Ma, F., Cholewa, E. W. A., Mohamed, T., Peterson, C. A., & Gijzen, M. (2004). Cracks in the palisade cuticle of soybean seed coats correlate with their permeability to water. *Annals of Botany, 94*(2), 213–228.
Meng, Y., Qu, G., Wang, T., Sun, Q., Liang, D., & Hu, S. (2017). Enhancement of germination and seedling growth of wheat seed using dielectric barrier discharge plasma with various gas sources. *Plasma Chemistry and Plasma Processing, 37*(4), 1105–1119.
Misra, N. N., Schlüter, O., & Cullen, P. J. (2016). Plasma in food and agriculture. *Cold plasma in food and agriculture: Fundamentals and applications* (pp. 1–16). Academic Press.
Mitra, A., Li, Y. F., Klämpfl, T. G., Shimizu, T., Jeon, J., Morfill, G. E., & Zimmermann, J. L. (2014). Inactivation of surface-borne microorganisms and increased germination of seed specimen by cold atmospheric plasma. *Food and Bioprocess Technology, 7*(3), 645–653.
Mittler, R., Vanderauwera, S., Gollery, M., & Van Breusegem, F. (2004). Reactive oxygen gene network of plants. *Trends in Plant Science, 9*(10), 490–498.
Moisan, M., Barbeau, J., Moreau, S., Pelletier, J., Tabrizian, M., & Yahia, L. H. (2001). Low-temperature sterilization using gas plasmas: A review of the experiments and an analysis of the inactivation mechanisms. *International Journal of Pharmaceutics, 226*(1–2), 1–21.
Noriega, G., Santa Cruz, D., Batlle, A., Tomaro, M., & Balestrasse, K. B. (2012). Heme oxygenase is involved in the protection exerted by jasmonic acid against cadmium stress in soybean roots. *Plant Growth Regulation, 31*, 79–89.
Puntarulo, S., Galleano, M., Sanchez, R. A., & Boveris, A. (1991). Superoxide anion and hydrogen peroxide metabolism in soybean embryonic axes during germination. *Biochimica et Biophysica Acta - General Subjects, 1074*(2), 277–283.
Quebedeaux, B., Sweetser, P. B., & Rowell, J. C. (1976). Abscisic acid levels in soybean reproductive structures during development. *Plant Physiology, 58*(3), 363–366.
Randeniya, L. K., & de Groot, G. J. (2015). Non-thermal plasma treatment of agricultural seeds for stimulation of germination, removal of surface contamination and other benefits: A review. *Plasma Processes and Polymers, 12*(7), 608–623.
Rupe, J., & Luttrell, R. G. (2008). Effect of pests and diseases on soybean quality. *Soybeans: Chemistry, production, processing, and utilization* (pp. 93–116). AOCS Press.
Sánchez, M. C., Ridao, A. D. C., & Colavita, M. L. (2015). *Diaporthe caulivora*: agente causal de cancro del tallo predominante en cultivos de soja del sudeste bonaerense. *FAVE, 14*(2), 1–20.
Santa-Cruz, D., Pacenza, N., Zilli, C., Pagano, E., Balestrasse, K., & Yannarelli, G. (2017). Heme oxygenase up-regulation under ultraviolet-B radiation is not epigenetically restricted and involves specific stress-related transcriptions factors. *Redox Biology, 12*, 549–557.
Sauer, M., Robert, S., & Kleine-Vehn, J. (2013). Auxin: Simply complicated. *Journal of Experimental Botany, 64*(9), 2565–2577.
Schupp, R., & Rennenberg, H. (1988). Diurnal changes in the glutathione content of spruce needles (*Picea abies* L.). *Plant Science, 57*(2), 113–117.
Sélcuk, M., Oksuz, L., & Basaran, P. (2008). Decontamination of grains and legumes infected with *Aspergillus spp.* and *Penicillum spp.* by cold plasma treatment. *Bioresource Technology, 99*(11), 5104–5109.
Shama, G., & Kong, M. G. (2012). Prospects for treating foods with cold atmospheric gas plasmas. *Plasma for bio-decontamination, medicine and food security* (pp. 433–443). Dordrecht: Springer.
Shu, K., Qi, Y., Chen, F., Meng, Y., Luo, X., Shuai, H., & Yang, F. (2017). Salt stress represses soybean seed germination by negatively regulating GA biosynthesis while positively mediating ABA biosynthesis. *Frontiers in Plant Science, 8*, 1372.
Singh, D., & Mathur, S. B. (2004). *Histopathology of seed-borne infections.* Washington DC: CRC Press.
Stolárik, T., Henselová, M., Martinka, M., Novák, O., Zahoranová, A., & Černák, M. (2015). Effect of low-temperature plasma on the structure of seeds, growth and metabolism of endogenous phytohormones in pea (*Pisum sativum* L.). *Plasma Chemistry and Plasma Processing, 35*(4), 659–676.
Sun, P., Sun, Y., Wu, H., Zhu, W., Lopez, J. L., Liu, W., & Fang, J. (2011). Atmospheric pressure cold plasma as an antifungal therapy. *Applied Physics Letters, 98*(2), 021501.
Terré, E. Balance de oferta y demanda de soja en Argentina. Available online: www.bcr.com.ar/Pages/Publicaciones/informativosemanal_noticias.aspxpIDNoticia=958. Accessed 30.02.2018. (Spanish) (2018).
Tong, J. Y., He, R., Zhang, X. L., Zhan, R. T., Chen, W. W., & Yang, S. Z. (2014). Effects of atmospheric pressure air plasma pretreatment on the seed germination and early growth of *Andrographis paniculata*. *Plasma Science and Technology, 16*, 260–266.
Van Loon, L. C., Geraats, B. P. J., & Linthorst, H. J. M. (2006). Ethylene as a modulator of disease resistance in plants. *Trends in Plant Science, 11*, 184–191.
Wang, S., Park, Y. S., Yang, Y., Borrego, E. J., Isakeit, T., Gao, X., & Kolomiets, M. V. (2017). Seed-derived ethylene facilitates colonization but not aflatoxin production by *Aspergillus flavus* in maize. *Frontiers in Plant Science, 8*, 415.
Wojtyla, L., Lechowska, K., Kubala, S., & Garnczarska, M. (2016). Different modes of hydrogen peroxide action during seed germination. *Frontiers in Plant Science, 7*, 66.
Wong-Ekkabut, J., Xu, Z., Triampo, W., Tang, I. M., Tieleman, D. P., & Monticelli, L. (2007). Effect of lipid peroxidation on the properties of lipid bilayers: A molecular dynamics study. *Biophysical Journal, 93*(12), 4225–4236.
Zhang, J. J., Jo, J. O., Huynh, D. L., Mongre, R. K., Ghosh, M., Singh, A. K., ... Jeong, D. K. (2017). Growth-inducing effects of argon plasma on soybean sprouts via the regulation of demethylation levels of energy metabolism-related genes. *Scientific Reports, 7*, 41917.
Zhang, Q., Sun, P., Feng, H., Wang, R., Liang, Y., Zhu, W., & Fang, J. (2012). Assessment of the roles of various inactivation agents in an argon-based direct current atmospheric pressure cold plasma jet. *Journal of Applied Physics, 111*(12), 123305.
Zhang, X., Liu, D., Zhou, R., Song, Y., Sun, Y., Zhang, Q., & Yang, S. Z. (2014). Atmospheric cold plasma jet for plant disease treatment. *Applied Physics Letters, 104*(4), 043702.
Zivkovic, S., Puac, N., Giba, Z., Grubisic, D., & Petrovic, Z. L. (2004). The stimulatory effect of non-equilibrium (low temperature) air plasma pretreatment on light-induced germination of *Paulownia tomentosa* seeds. *Seed Science and Technology, 32*, 693–701.