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

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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 minutes to a quasi-stationary (50 Hz) dielectric barrier discharge plasma operating at atmospheric pressure air. Different carrying gases (O₂ and N₂) 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.

Keywords:

Non-thermal plasma

Diaporthe/Phomopsis complex*Glycine max*

Seed quality

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 loingicola*) (Sánchez, Ridaó, & 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 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 (Laroussi & Leipold, 2004; Fridman et al., 2008; 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 (Moisan et al., 2001; Ma et al., 2004). 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 (Sun et al., 2011; Daeschlein et al., 2014), food contaminant fungi (Dasan, Mutlu, & Boyaci, 2016; Basaran, Basaran-Akgul, & Oksuz, 2008) 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 (Tong et al., 2014; Ling et al., 2014; Stolarik et al., 2015; Ji et al., 2016; Meng et al., 2017) without provoking changes in the gene structure of the seeds as they are based on non-ionizing low level radiation (Zivkovic et al., 2004; Jiafeng et al., 2014; Randeniya & Groot, 2015). 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 analysed 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).

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 1.2mm-thick Thernofase barrier and 85 W for the 2.5mm-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 2.5mm-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 exhibit the diffuse mode at the negative voltage cycle. It is expected that the discharge mode of the DBD has influence on the production of the reactive species in the plasma (Brandenburg, 2017). The current pulses shown in Fig. 2 are related to the presence of thin plasma filaments bridging the electrode gas gap (filament radius $\sim 50\text{-}100\ \mu\text{m}$) with current densities in the range $100\text{ to }1000\ \text{A cm}^{-2}$, exhibiting a chaotic spatio-temporal behavior. The electron density n_e and temperature T_e are in the $10^{20}\text{-}10^{21}\ \text{m}^{-3}$ and $1\text{-}10\ \text{eV}$ ($1\ \text{eV} = 11600\ \text{K}$) ranges inside a plasma filament, while n_e and T_e are in the $10^{15}\text{-}10^{17}\ \text{m}^{-3}$ and $0.2\text{-}5\ \text{eV}$ ranges for a diffusive discharge. The short lifetime of the plasma filaments ($1\text{-}10\ \text{ns}$) prevents the overheating of the discharge ($< 10\ \text{°C}$ in atmospheric pressure air), being the gas temperature T_g in the filament close to the background gas $\sim 300\ \text{K}$ (Fridman, Chirokov, & Gutsol, 2005; Brandenburg, 2017). 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 $65\ \text{W}$ for the $1.2\ \text{mm}$ thick Thernofase barrier and $85\ \text{W}$ for the $2.5\ \text{mm}$ thick Pertinax sheet with 2 Mylar films of $100\ \mu\text{m}$ 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.

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 ($25 \pm 2\ \text{°C}$ in the dark). After 7 days, the percentage of seeds infected with D/P complex (D/P %) was calculated.

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

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 $25\ \text{°C}$, light/dark (12 h/12 h) and within 7 days (ISTA, 2006).

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

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 $80\ \text{°C}$ 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 & Anderson (1973).

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

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

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

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

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

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).

$$(7) \text{ Water uptake (\%)} = (m_t - m_0) / m_0 * 100$$

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 minutes 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 3000g for 20 min and the resulting supernatants were used for malondialdehyde (MDA) determination according to Heath & Parker (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 minutes, centrifuged at 3500 rpm for 10 minutes. 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 ($155 \text{ mM}^{-1} \text{ cm}^{-1}$). Results were expressed as nmol MDA g tissue⁻¹.

2.9. *In situ localization of H₂O₂*

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. H₂O₂ was visualized as deposits of dark brown stain (Noriega et al., 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 13000g for 30 minutes 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, & Boveris (1979). The enzyme assay contained 2 mM H₂O₂ 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 H₂O₂ at 240 nm. Results were expressed in $\mu\text{mol min}^{-1} \text{ mg prot}^{-1}$. Superoxide dismutase (SOD) activity was measured at 25°C according to Becana, Aparicio-Tejo, Irigoyen, & Sanchez-Diaz (1986). The reaction mixture contained 2.2 μM riboflavine, 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 minutes and stopped by switching the light off. The reduction in NBT was followed by reading absorbance at 560 nm every 2 minutes. 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 & Kamínek (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 et al. (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 \pm 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). Dunett'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 \pm 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 (Tong et al., 2014; Li et al., 2017), 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 et al., 2014; Tong et al., 2014; Ling et al., 2014; Mitra et al., 2014; Meng et al., 2017) or for controlling food surface microorganisms (Laroussi & Leipold, 2004; Mitra et al., 2014; Dasan, Mutlu, & Boyaci, 2016) 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 (Thermax and Pertinax+Mylar), two gases (O₂ and N₂) and three times of exposure (one, two and three minutes). Significant differences were detected between IC and treatments PMN2, PMN3, PMO2, PMO3, TN1 y 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 et al. (2008), Dasan, Mutlu, & Boyaci (2016), Iseki et al. (2010), Ishikahua 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; Tong et al., 2014; Ling et al., 2014; Stolarik et al., 2015; Dobrin, Magureanu, Mandache, & Ionita, 2015; Ji et al., 2016; Li et al., 2017; Meng et al., 2017).

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 (Rupe & Luttrell, 2008; Li, 2011). 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 (Ling et al., 2014; Stolarik et al., 2015; Dobrin, Magureanu, Mandache, & Ionita, 2015; Ji et al., 2016; Li et al., 2017; Meng et al., 2017) 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, Grynyov, Bormashenko, & Drori, 2012; Li et al., 2017; Stolarik et al., 2015; Ji et al., 2016) 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, Grynyov, Bormashenko, & Drori (2012) in lentils, beans and wheat.

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 (Balestrasse, Gardey, Gallego, & Tomaro, 2001; Mittler, Vanderauwera, Gollery, & Van Breusegem 2004; Apel & Hirt, 2004; Bustingorri, Balestrasse, & Lavado, 2015; 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, Schlüter, & Cullen, 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).

According to Puntarulo, Galleano, Sanchez, & Boveris (1991), the presence of H_2O_2 in germinating soybean seeds achieves the highest level at six hours 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 colour intensity at six hours indicated lower content of H_2O_2 in the IC than in treated seeds and the HC. Moreover, at twelve hours, treated seeds presented significant reductions in peroxide content compared with IC (which explain the negative values of variation on colour intensity). Our results coincided with Puntarulo, Galleano, Sanchez, & Boveris (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 (Fig. 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, Galleano, Sanchez, & Boveris, 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.

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, & Rowell (1976), Fountain & Bewley (1976), Ackerson (1984) and Brenner & 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, & 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 et al. (2010, 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 (Jhonson and 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) 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, 2011) proposed that H₂O₂ 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 orchestrated by H₂O₂), 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.

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Tables

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
Thermafase	N ₂	1	TN1
		2	TN2
		3	TN3
	O ₂	1	TO1
		2	TO2
		3	TO3
Infected control			IC
Healthy control			HC

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

Treatment	Thiobarbituric acid reactive substances (nmol Malondialdehyde g tissue ⁻¹)					
	<i>Dry seed</i>			<i>Imbibed seed</i>		
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 (Dunett's test, P < 0.05).

Table 3. Percentage of variation on the color intensity of stain deposits of H₂O₂ on seeds in comparison with IC at different times of imbibition.

Time of imbibition (hour)	Variation on colour 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%

Figure captions

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

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

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, $P < 0.05$).

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 minutes 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, $P < 0.05$).

Fig. 7. In situ localization of H_2O_2 along twenty-four hours of seed imbibition.

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, $P < 0.05$).

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 h (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, $P < 0.05$).

Highlights

- Seed infection with *Diaporthe/Phomopsis* fungi is reduced by 49-81% after plasma treatment
- Exposure of soybean seeds to plasma treatment enhances their quality (sanity, germination and vigor)
- Plasma treatments *per se* do not trigger oxidative damage to seeds compared with the healthy control

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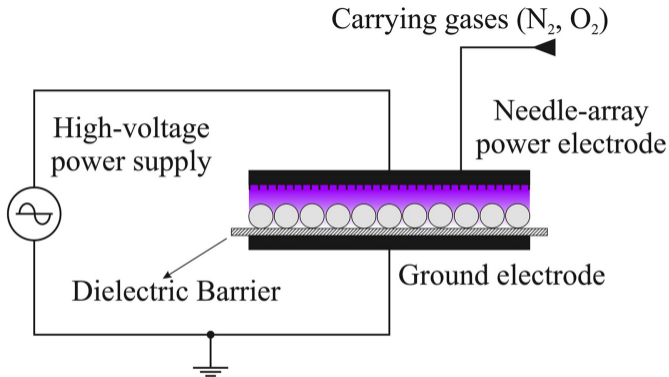


Figure 1

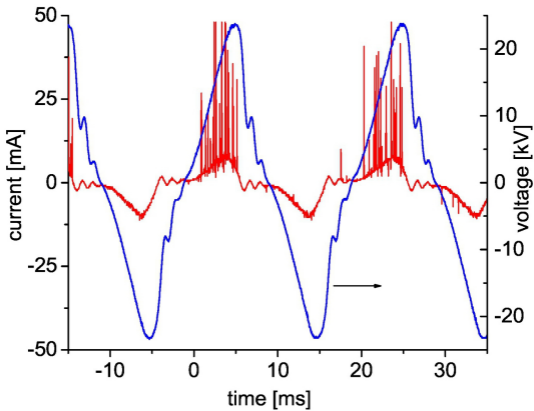


Figure 2

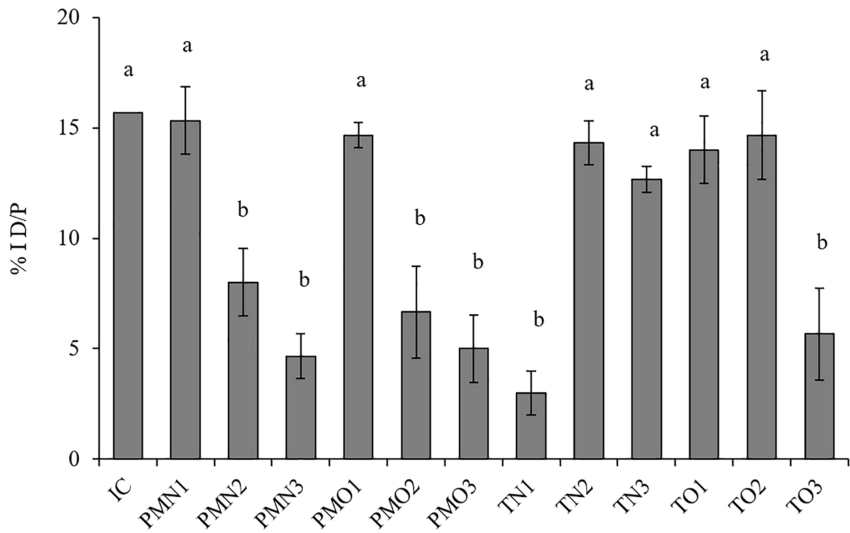


Figure 3

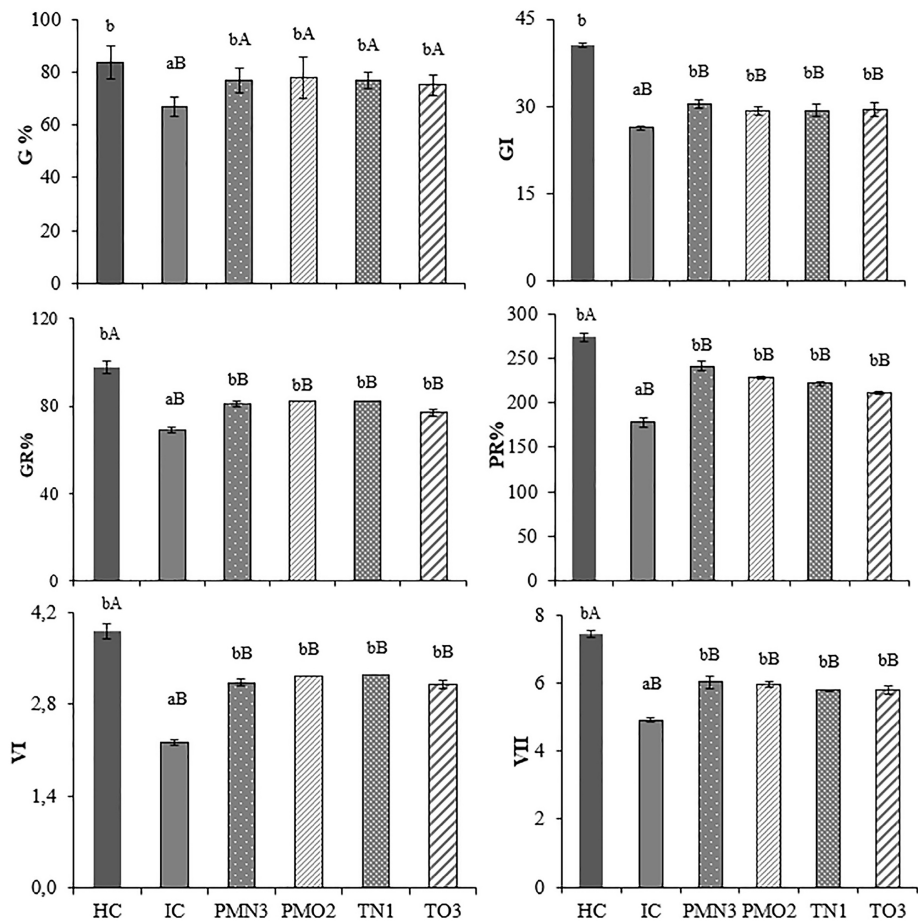


Figure 4

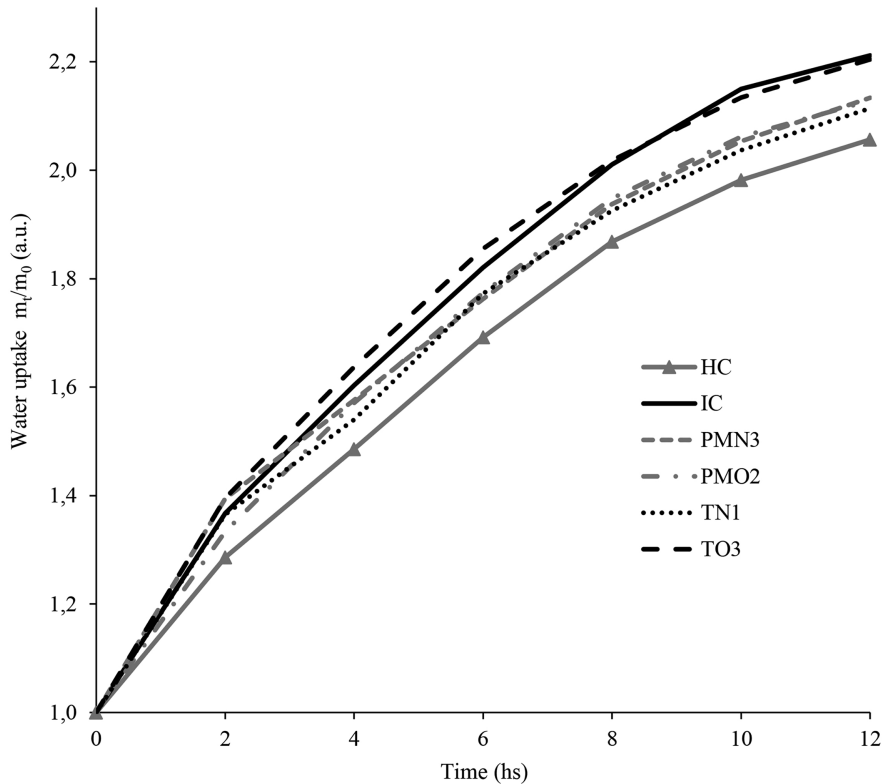


Figure 5

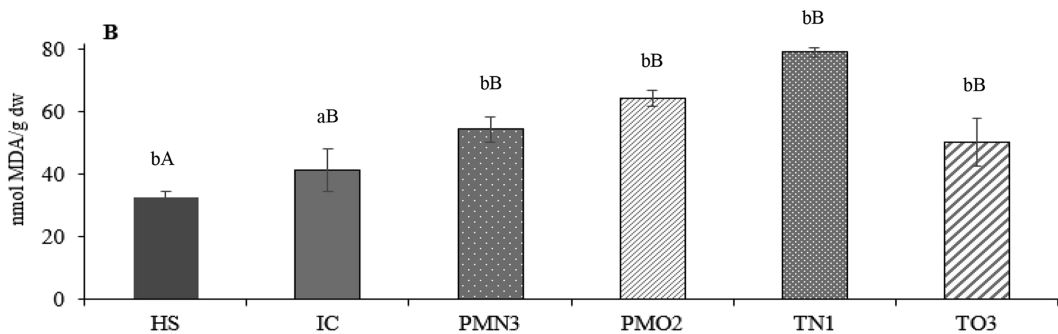
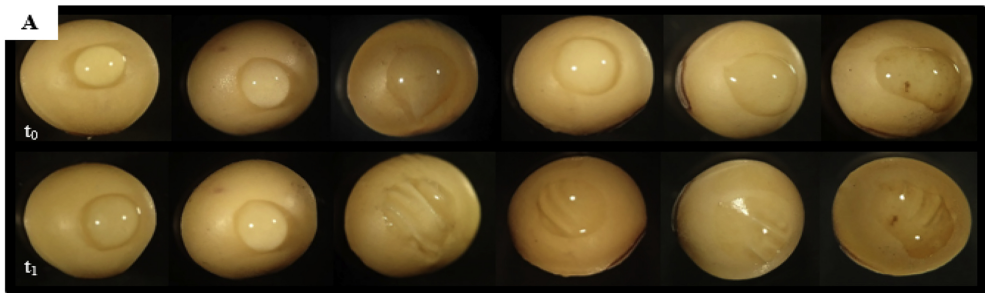


Figure 6

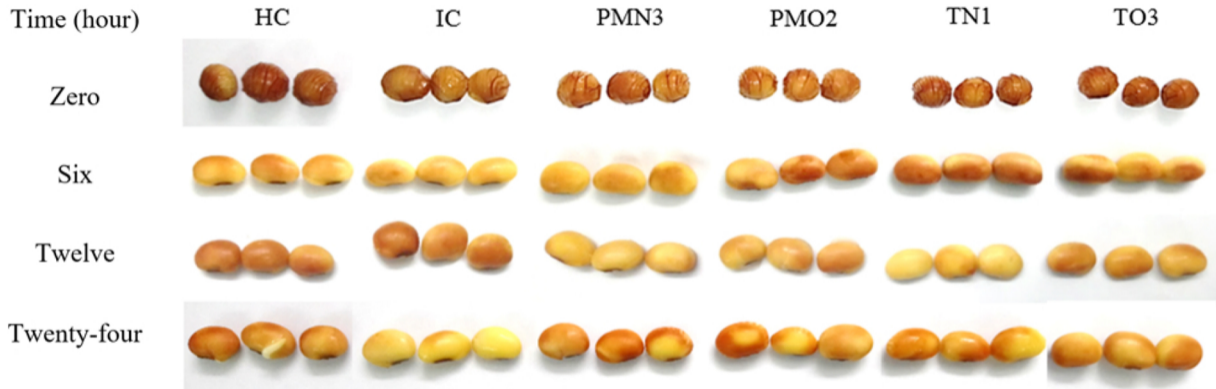


Figure 7

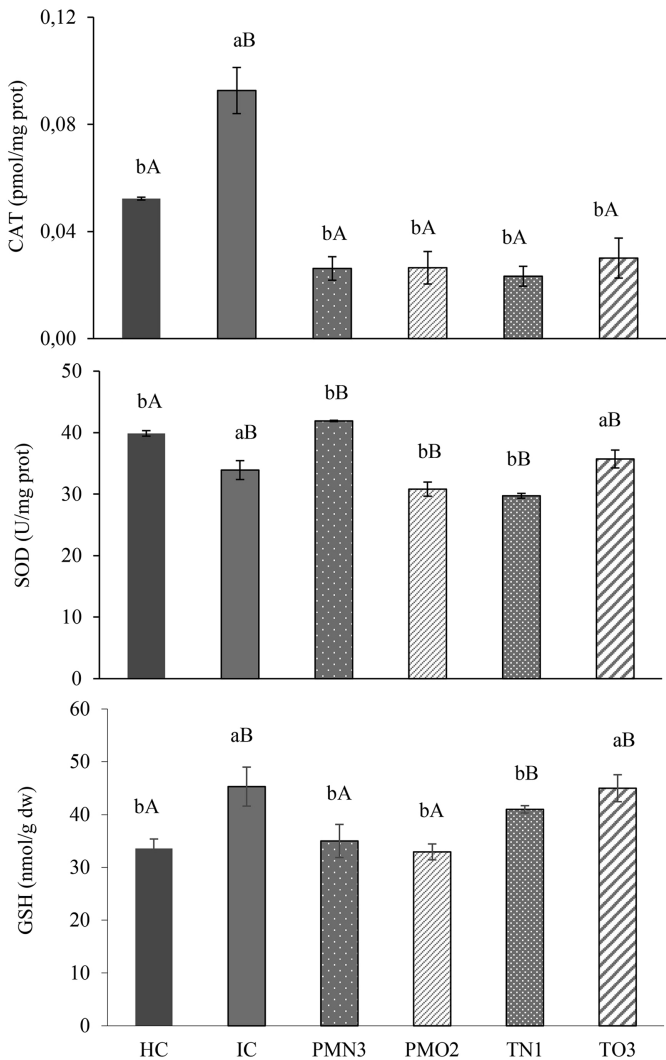


Figure 8

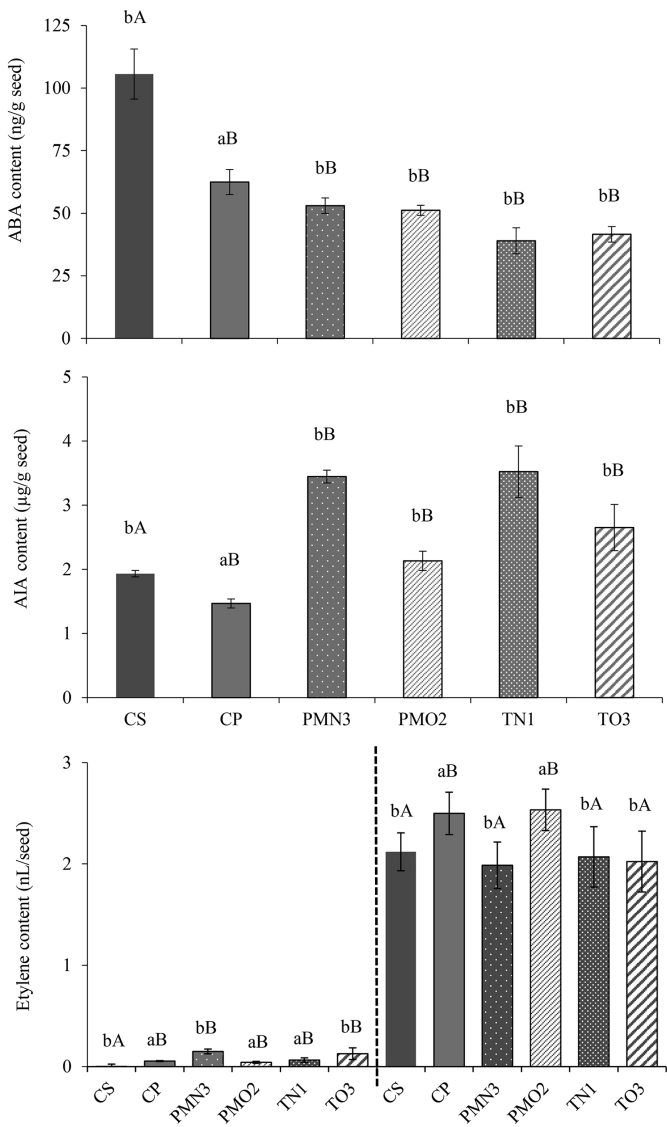


Figure 9