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Non-Thermal Plasmas Affect Plant Growth and DNA Methylation Patterns in *Glycine max*

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Abstract

Non-thermal plasmas (NTP) are partially ionized gases that represent a promising technology for seed treatment to enhance seed health while promoting germination and vigor in a fast, cost-effective, and eco-friendly way. The seed treatment with NTP generates phenotypic variations in plants that could be related to changes in DNA methylation. This work analyses the effects of two different NTP: nitrogen for 3 min (PMN3) and oxygen for 2 min (PMO2) applied to soybean (*Glycine max*) seeds. Growth parameters of plants grown from treated and untreated seeds were evaluated at two growth stages: 6 and 20 days after sowing (DAS). MSAP (Methylation Sensitive Amplified Polymorphism) markers were assayed to evaluate epigenetic changes induced by NTP treatments. Plants obtained from PMN3 and PMO2-treated seeds were phenotypically similar to each other: exhibited a superior growth at both stages. At 6 DAS root and shoot length and fresh weight surpassed the Control, while at 20 DAS root length and fresh and dry weight were higher than Control. PMN3 and PMO2 induced DNA methylation changes with respect to the Control plants, with higher differentiation at 20 DAS than at 6 DAS. The epigenetic variability and the phenotypic variability correlated only at 20 DAS ($R^2 = 0.5$). The observed phenotypic differences among Control and NTP-treated plants could not be explained by overall changes in the methylation levels, but both demethylation and methylation changes at specific loci appear to be operating in response to NTP treatments.

Keywords MSAP · Epigenetics · Soybean · Non-thermal plasma

Introduction

Non-thermal plasmas (NTP) are partially ionized (quasi-neutral) gases, composed of molecules, atoms, ultraviolet (UV) photons, highly energetic electrons, charged particles, and reactive species. In particular, highly reactive species of nitrogen, oxygen, and hydrogen (RNS, ROS, and RHS, respectively; Moreau et al. 2008; Vandamme et al. 2012;

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Hertwig et al. 2018) are formed when ambient air or a similar gas mixture is used as the plasma gas. According to Panngom et al. (2014), the functional diversity of the different reactive species that compose the NTP, allows them to be used for solving various biological problems such as inactivation of microorganisms and cancer cells, promotion of wound healing and tissue regeneration, decontamination of seeds and improvement of seed germination and plant growth, control of fungal pathogens infecting crops and enhancement of plant resistance to fungal infections. In recent years, NTP have been shown to modify plant phenotype and to stimulate germination and early growth of seedlings of different species, when applied to seeds before sowing (Jiayun et al. 2014; Li et al. 2017; Hosseini et al. 2018; Yodpitak et al. 2019). In this sense, great efforts are being made worldwide to explain how NTP exert their effects on seeds and on the plants that grow from them (Ling et al. 2014; Ji et al. 2016; Li et al. 2017; Hosseini et al. 2018). However, the knowledge about this topic is still incipient and multiple mechanisms seem to be involved in such effects. It has been suggested that active plasma particles (particularly ROS and RNS) and UV radiation can penetrate through the seed coat and cause redox reactions within cells, influencing germination and early growth of plants (Considine et al. 2014; Jiayun et al. 2014). Zhang et al. (2017) explored gene regulation as a possible mechanism of action of NTP when used for soybean treatment. These authors concluded that the stimulating effect of NTP on plant growth responds to the combination of three mechanisms that are initiated from the exposure of seeds to the plasma atmosphere: (i) increased soluble protein concentrations, (ii) increased activity of antioxidant enzymes and (iii) demethylation of genes related to early metabolism.

Phenotypic differences between individuals may respond to different sources of variability. Among them, the modification in the DNA sequence represents the most stable type of variation since it is irreversible and inheritable (Medrano et al. 2014). Another source of variability is the epigenetic variation and includes changes in genetic material (such as DNA methylation and histones modifications) that do not affect the sequence of genes and that may be caused by signals that are external to the individual where the modifications occur (Richards et al. 2010; Medrano et al. 2014). These epigenetic changes determine which genes are expressed, thus shaping the phenotype of an organism, while the sequence of its DNA remains intact (Jablonka et al. 2009). The epigenetic variations are reversible and are part of the molecular processes that underlie the phenotypic variability that can be observed as a response to variations in the surrounding environment (Richards et al. 2010). Also, it has been shown that some epigenetic patterns persist in gametes and can be transmitted to the following generations (Kakutani 2002; Calarco et al. 2012; Walker et al. 2018) increasing the tolerance of the progeny to certain stress situations (Kou et al. 2011; Ou et al. 2012). Various authors (Aina et al. 2004; Dowen et al. 2012; Baulcombe et al. 2014; Meyer 2015; Deleris et al. 2016; Verhoeven et al. 2016) have documented epigenetic changes induced by stress conditions either biotic (herbivores or pathogens) or abiotic (drought, osmotic stress, extreme temperatures). In this sense, epigenetic variation seems to play an important role in the processes of rapid acclimatization of organisms to the environment and may give rise to new phenotypes (Cara et al. 2013; Ibañez et al. 2021; Varela et al. 2020). The variability in DNA methylation patterns and cytosine methylation levels can be studied by employing the Methylation Sensitive Amplification Polymorphism (MSAP) technique, through methylation sensitive restriction enzymes (such as *Hpa*II and *Msp*I isoschizomers that recognize 5′-CCGG-3′ sites), and subsequent adapter ligation and PCR amplification (Xiong et al. 1999; Gimenez et al. 2016).

Although it has been widely demonstrated that seed treatment with NTP generates phenotypic variations in the plants that grow from them, there are currently few studies exploring overall changes in the DNA methylation or its possible implications in the origin of these phenotypic variations. In this sense, the present work aimed to detect phenotypic variations in soybean plants (6 and 20 days after sowing) grown from seeds treated with NTP and to analyze with MSAP markers the possible role of epigenetic variability in the origin of the observed phenotypic differences.

Material and Methods

Plant Material and Non-Thermal Plasma Treatment

Soybean seeds (DM 53i53 IPRO) of high quality (completely free of pathogens, germination power > 85%) were provided by Don Mario Semillas S.A. These seeds were exposed to NTP, using a 2.5 mm thick Pertinax sheet with 2 Mylar films of 100 µm thickness each. Nitrogen or oxygen gas (purity above 99.5%) was injected into the unconfined discharge region as carrier gas with a (measured) gas flow rate of 6 standard liters per minute (slm). Exposure times of 3 and 2 min were used for nitrogen and oxygen respectively, leading to PMN3 and PMO2 treatments. These plasma treatments were selected according to the results obtained in our previous works (Pérez-Pizá et al. 2019; 2020) where we found significant enhancement of soybean growth and nodulation through the combination of gas N_2, Pertinax and Mylar barrier and 3 min of exposure (treatment PMN3) and gas O_2, Pertinax and Mylar barrier and 2 min of exposure (treatment PMO2). The dielectric barrier discharge (DBD) plasma source used for seed treatment was described in detail in Pérez-Pizá et al.

(2018). The voltage of the discharge under the processing conditions was measured using a 1:1000 high–impedance voltage probe (Tektronix P6015A) while the integral of the discharge current was measured from the voltage across a capacitor (0.5 F) connected in series to the ground electrode of the discharge. The waveforms were recorded using a 4–channel oscilloscope (Tektronix TDS 2004C). The corresponding Lissajous figures (Pipa et al. 2012) of the discharge are shown in Supplemental Fig. S1. Within the experimental errors the discharge power consumptions are quite similar for both carrier gases (about 16 W), thus showing that the electron attachment processes with oxygen molecules are not relevant under the conditions considered.

Ozone concentration in the discharge region was also measured by UV absorption spectroscopy. The light of the UV source (Avantes AvaLight–CAL–Mini) was collimated by a UV optical system and directed through the discharged effluent. A photomultiplier (Hamamatsu R6350) attached to a monochromator (OBB, grating 2400 lines mm^{-1}, blazed at 300 nm) converted the incoming light signal into an electrical signal. The intensity of UV light at 254 nm was used to calculate ozone density based on Beer's law. The photoabsorption cross-section used was 1.147×10^{-21} m^2 (Daumont et al. 1992). Supplemental Fig. S2 shows the temporal evolution profiles of ozone density under the processing conditions for the two carrier gases. Measurements represent mean values of ozone density over the diameter (130 mm) of the power electrode. The time resolution and detection limit of the measurements were about 0.5 s and 3 ppm, respectively. In both cases the ozone density increased during the first 25 s until reaching stationary values. However, as expected, the concentration plateau (around 70 ppmv) exhibited by the oxygen is considerably higher than that of nitrogen (around 20 ppmv) (Kossyi et al. 1992). It is important to note that despite other reactive species concentrations that could not be measured (because both the absorption cross-sections and the concentrations are relatively much lower than that of O_3), a small overestimation in the measured O_3 concentration may still be expected due to the overlap of absorbance from other low–concentration air species (as NO_2 and N_2O_4) over the 254 nm wavelength (Moiseev et al. 2014).

Seeds were placed in the region of the active plasma on the dielectric barrier. During the exposure (at half the exposure time), seeds were mixed mechanically to ensure uniform contact between them and plasma. The temperature of the seeds was measured with an IR handheld thermometer. It never exceeded 38 °C during the experiments. In the end, seeds were removed from the discharge region and stored in sterilized containers in a cold chamber (5 °C). A group of seeds was kept without exposure to plasma, constituting the Control.

Plant Growth Conditions

Seeds exposed to non-thermal plasma (PMN3 and PMO2) and Control were sown in trays containing fine wet sand (60% of field capacity) and, in parallel, in plastic pots (1 L) containing perlite. Trays and pots were randomly distributed in a growth chamber with a 12 h photoperiod (light/dark), 25 °C temperature and photosynthetic photon flux density of 350 μE m^{-2} s^{-1}. Pots were watered every 3 days with Hoagland solution prepared according to Leggett et al. (1971), using half of the nitrogen dose recommended by the authors. Trays were removed from the chamber 6 days after sowing (DAS), while pots remained inside until day 20 after sowing. Phenotypic and epigenetic variabilities were assessed at two stages of growth: 6 DAS and 20 DAS. In each stage, three replicates (of ten plants each) were generated ($n = 3$), thus evaluating a total of 30 plants per treatment.

Phenotypic Variability: Evaluation of Biometric Parameters

Root and shoot length (cm) were measured using a tape measure. Shoots (stem + leaves) and roots were weighed and then dried at 80 °C for 120 h to obtain its dry weight (g). Fresh and dry weights (FW and DW, respectively) were recorded using an analytical balance (0.001 g).

Epigenetic Variability: MSAP Analysis

After evaluating the biometric parameters, the meristematic apexes were collected from the plants at 6 DAS and from the plants at 20 DAS. Each sample was grounded using a mortar and liquid nitrogen. Total genomic DNA was extracted using the CTAB (Hexadecyltrimethylammonium Bromide) extraction protocol according to modifications from Varela et al. (2020). Briefly, CTAB buffer solution presented 2% CTAB (v/v), 2 M Tris HCl pH 8, 0.5 M EDTA pH 8, 2.5 M NaCl, 1% polyvinylpyrrolidone and 0.4% (v/v) 2-βmercaptoethanol. Two DNA precipitations were made with chloroform/isoamyl alcohol (24:1) and one more with isopropanol (98.5%). The precipitated DNA was rehydrated in Milli-Q water and quantified by spectrophotometric absorbance at 260 nm. The integrity of the DNA obtained was assessed by loading 100 ng on a 0.8% agarose gel.

The MSAP technique was applied following the protocol described by Cara et al. (2013). Two selective fluorescent-labeled **Eco*RI primers (FAM) were combined with three *Hpa*II/*Msp*I primers (Supplemental Table S1). The amplification products were separated by capillary electrophoresis

on an automatic fragment analyzer (Genetic Analyzer 3130, Applied Biosystems, CA, USA) and analyzed with GeneMaker v2.7.0 (SoftGenetics, PA, USA) taking into account sizes between 100 and 600 bp. To determine the presence/absence of fragments, a threshold of 50 relative fluorescent units (rfu) was considered. Each locus obtained with *Eco*RI/*Hpa*II and *Eco*RI/*Msp*I combination presents multistate information (Schulz et al. 2013). Fragments obtained in the *Hpa*II but absent in the *Msp*I lane and vice versa were considered as methylated states and fragments presented in both lanes were considered as non-methylated states (Ibañez et al. 2021). Finally, the absence of fragments in both lanes gives rise to an ambiguous interpretation, since its presence could be due to complete methylation or to a mutation in the nucleotide sequence of the restriction site (Schulz et al. 2013). According to the fragment presence/absence at each locus (Supplemental Fig. S3), a binary matrix was constructed. The sequences of adapters and primers used, as well as the number of polymorphic fragments obtained for each primer combination used in the amplification, are shown in Supplemental Table S1.

Data Analysis

All analyses were performed with R version 4.0.2 (R Core Team 2020). Biometric parameters (length, dry and fresh weight of roots and shoots) were analyzed with ANOVA, and principal component analysis (PCA) was performed to determine associations between treatments and biometric parameters. The PCA was performed with centered and variance-scaled data using 'FactoMineR' and 'factoextra' packages in R (Le et al. 2008; Kassambara et al. 2017). For MSAP data, a principal coordinate analysis (PCoA) was performed to determine the relationships between the NTP treatments and the Control. All analyses were performed with polymorphic markers, while uninformative markers as monomorphic loci and singletons (i.e., fragments present only in one sample—Supplemental Fig. S3) were excluded (Varela et al. 2020). From the binary matrix, the epigenetic distances were calculated using the Sorensen–Dice coefficient (Marfil et al. 2019; Ibañez et al. 2021). The PCoA was realized with distance matrix, and the effect of the treatments was evaluated by analyzing the variance of the distance matrices with the adonis function of the R 'vegan' package (Oksanen et al. 2019). This function allows differentiating the groups mean through the partition of the sources of variability, and with permutation tests, it allows obtaining the significance of the partitions. Also, to compare the magnitude of changes in DNA methylation patterns between plasma treatments, a consensus methylation state for each epiloci was inferred based on the methylation patterns (MSAP epialleles) observed in the Control plants (Supplemental Fig. S3; Verhoeven et al. 2010; Marfil et al. 2019). The consensus state for each epiloci was established independently for plants at 6 and 20 DAS considering monomorphic epialleles within the Control plants and those that had only one deviating observation among the three replicate samples of each phenological stage. The consensus state could be established for 525 epialleles at 6 DAS and for 543 epialleles at 20 DAS. The frequency of plasma-induced methylation changes was analyzed with a GLM model, setting the marker and the individual sample effect as random factors (Marfil et al. 2019). The linear correlation between the distance matrices of epigenetic variability and phenotypic variability was evaluated by the Mantel test.

Results

Plants Grown from Control and Non-Thermal Plasma-Treated Seeds Presented Phenotypic Differences

Root and shoot biometric parameters were measured in soybean plants grown from Control and two non-thermal plasma treated (PMN3, PMO2) seeds at 6 and 20 DAS (Figs. 1, 2 and 3). The PCA analysis showed a general pattern in the variability of the treated plants that differ from the Control plants for both stages (Fig. 2). At 6 DAS (Fig. 2a), both NTP treatments were related to root FW and length and shoot length, while Control plants were related to shoot FW and DW. At 20 DAS, all traits measured were related to NTP treatment (Fig. 2b), indicating that plants

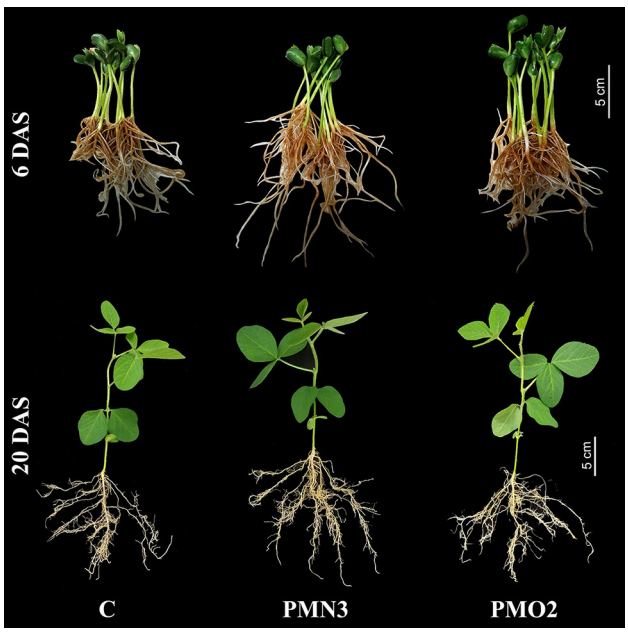

Fig. 1 General aspect of soybean plants under Control (C), PMN3 and PMO2 treatments at 6 days after sowing (DAS, upper panel) and 20 DAS (lower panel)

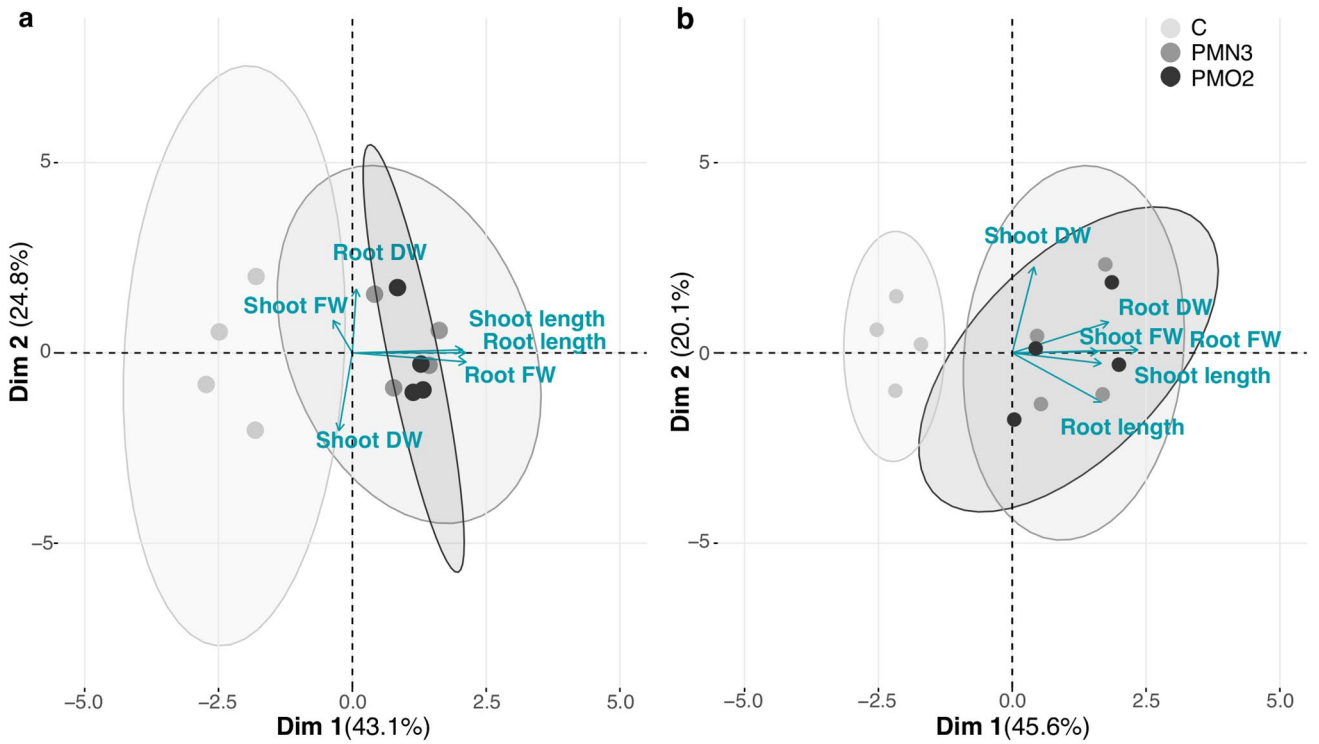

Fig. 2 Phenotypic variability measured in soybean plants treated with non-thermal plasma. Principal component analysis of shoot and root length, fresh and dry weights (FW and DW, respectively) measured at (**a**) 6 and (**b**) 20 days after sowing in plants under Control (C), PMN3 and PMO2 treatments

grown from NTP-treated seeds showed the greatest values of shoot and root length, FW and DW.

The ANOVA analysis showed no differences among treatments for shoot FW and DW in none of the two phenological stages (Fig. 3a and b). No significant differences in the six measured traits were detected between NTP treatments in none of the two phenological stages. Significant differences in shoot length between NTP treated and Control plants (Fig. 3c) were observed at 6 DAS: on average, NTP-treated plants were 14% longer than Control plants (6.86 cm vs 6.04 cm).

Regarding the three root parameters, significant differences were observed between plants grown from NTP treated and Control seeds at both phenological stages, except for root DW at 6 DAS. Plants grown from NTP-treated seeds, overcame Control plants in root FW and length (Fig. 3d and f). Moreover, we observed at 6 and 20 DAS stages, increases of 33% and 17%, respectively, on root FW (0.64 g vs 0.48 g at 6 DAS and 5.98 g vs 5.09 g at 20 DAS, respectively) and of 23% and 17% on root length (11.65 cm vs 9.43 cm at 6 DAS and 22.07 cm vs 18.89 cm at 20 DAS, respectively). Regarding root DW (Fig. 3e), only at 20 DAS plants grown from plasma-treated seeds outperformed Control plants (0.41 and 0.42 g vs 0.37 g).

The Stage of Growth and the Non-Thermal Plasma Treatments Influenced the Epigenetic Variability Observed

The three primer combinations used amplified a total of 267 fragments. 75 singletons and 9 monomorphic fragments were excluded from the analysis. The remaining 183 fragments were transformed into 206 polymorphic epialleles (Supplemental Fig. S3 and Fig. S4). When the whole matrix was analyzed, the first two axes in the principal coordinate analysis explained 28.9% of the total epigenetic variability (Fig. 4a). At both stages of growth, PMN3 and PMO2 treated plants overlapped each other and were differentiated from the Control plants. This differentiation between NTP treatments and the Control was higher at 20 DAS than at 6 DAS, principally along the first axis (Fig. 4a). These differences were confirmed by permutational analysis of variance (permanova) through the distance matrix: treatment and the stage of growth explained 14.9% ($P = 0.0049$) and 11.8% ($P = 0.0009$) of the epigenetic variability, respectively. To visualize the effect of the treatments, each stage of growth was analyzed separately. The first two axes explained 37.9% and 44.8% of the total epigenetic variability at the 6 and 20

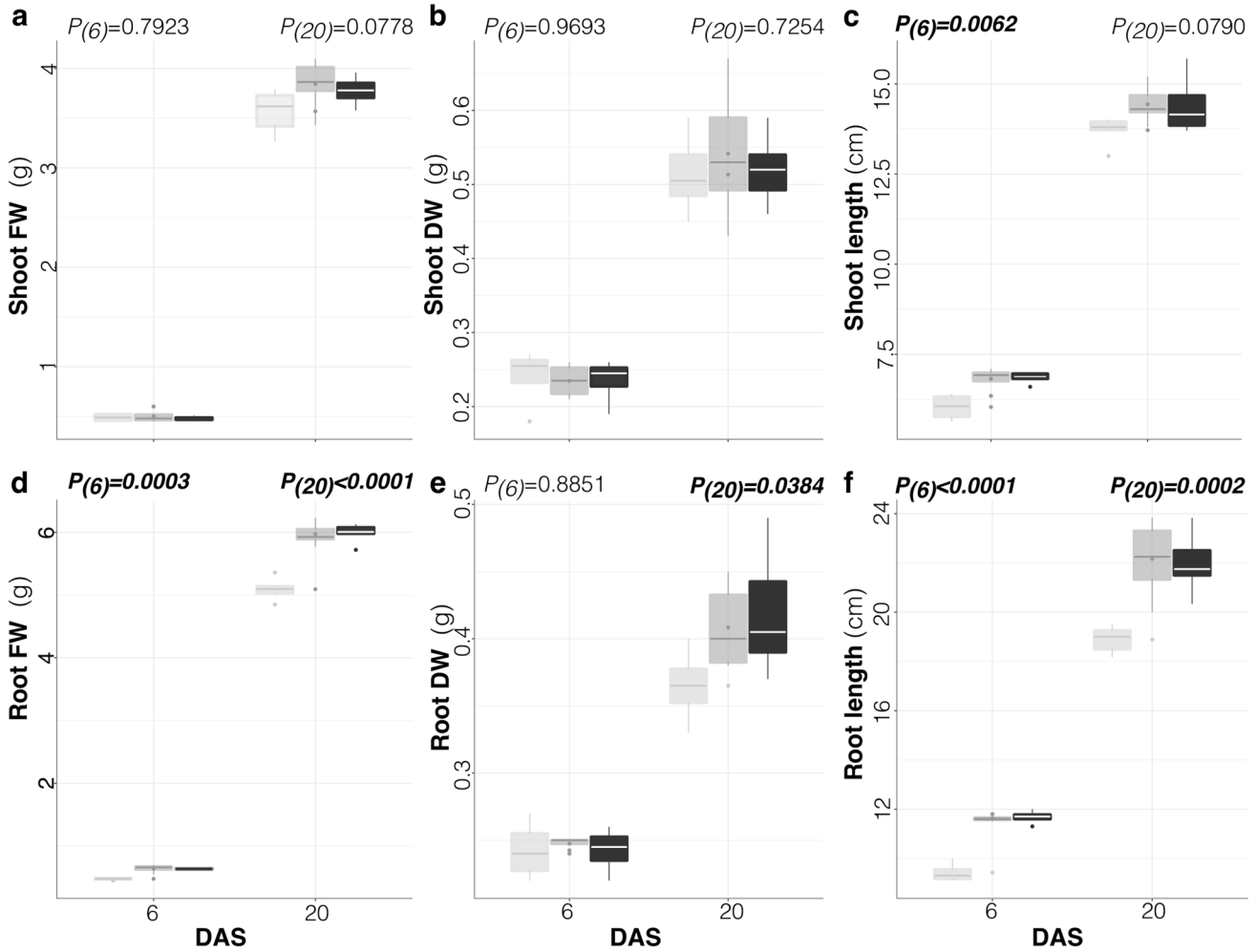

Fig. 3 Univariate analysis of variance for phenotypic traits measured in soybean plants treated with non-thermal plasma. Box plots based on (**a**) shoot fresh weight (shoot FW), (**b**) shoot dry weight (shoot DW), (**c**) shoot length, (**d**) root fresh weight (root FW), (**e**) root dry weight (root DW) and (**f**) root length, measured at 6 and 20 days after sowing, in plants grown from: Control seeds (C—light grey), seeds exposed to plasma for 3 min employing nitrogen as carrier gas (PMN3—grey) and seeds exposed to plasma for 2 min employing oxygen as carrier gas (PMO2—black)

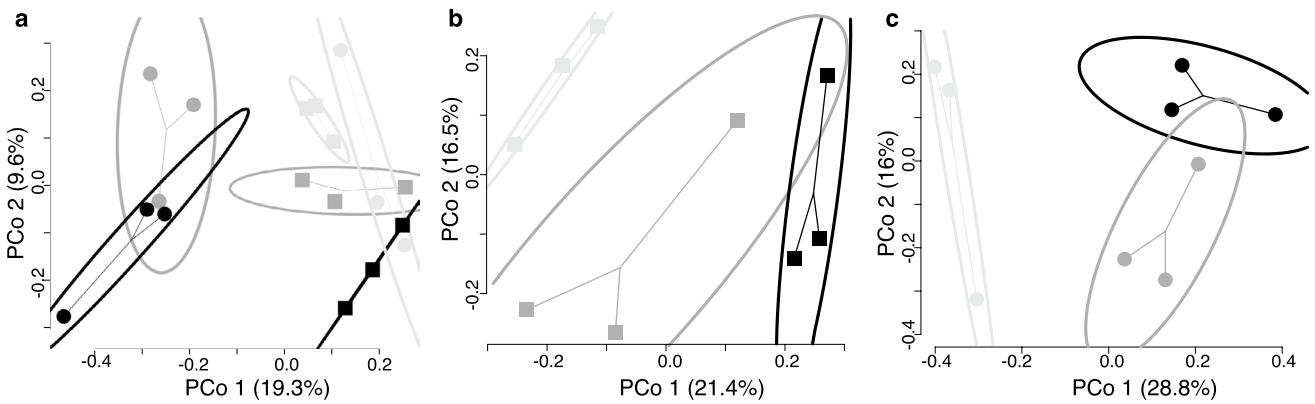

Fig. 4 Epigenetic variability measured in soybean plants treated with non-thermal plasma. Principal coordinate representation based on methylation sensitive amplified polymorphism (MSAP) markers evaluated in plants under Control (C—light grey), PMN3 (grey) and PMO2 (black) treatments, at (**a**) 6 (squares) and 20 (circles) days after sowing (DAS), (**b**) at 6 DAS and (**c**) at 20 DAS. Ellipses show a 0.90 confidence interval

DAS, respectively (Fig. 4b and c). Again, at both stages of growth, the confidence intervals of NTP-treated plants were overlapped and differentiated from Control plants. At 6 DAS, the PMN3 treatment induced higher dispersion among biological replicates than the observed for Control and PMO2-treated plants (Fig. 4b). At 20 DAS, PMN3 and PMO2-treated plants showed similar epigenetic differentiation with respect to the Control plants, principally along the first axis (Fig. 4c). Moreover, these differences were confirmed with permanova analysis: 33.6% and 40% ($P = 0.0129$ and $P = 0.0059$, respectively) of the variability was explained by NTP treatment at 6 and 20 DAS, respectively.

The proportion of epiloci that presented differences with respect to the consensus methylation patterns was higher in plants treated with NTP than in Control plants at both stages of growth (Table 1). The comparison with the consensus epigenotype confirmed the higher percentages of changes at 20 DAS than at 6 DAS. Also, pairwise contrasts between the Control plants (consensus methylation pattern) and individual NTP treatments showed that PMN3 and POM2-induced similar levels of consensus deviations at both stages of growth (Table 1).

Both, the stage of growth and the NTP treatments had influences on DNA methylation level (Table 2). In Control plants, methylated loci decreased at 20 DAS with respect to 6 DAS. At 6 DAS, PMO2 treatment had 22% more non-methylated loci than Control plants. On the other hand, at 20 DAS plants treated with PMN3 showed 17% less non-methylated loci than Control ones.

Epigenetic and Phenotypic Correlation was Observed Only at 20 DAS

Mantel tests showed correlation between phenotypic and epigenetic variability at 20 DAS ($R^2 = 0.5$; $P = 0.001$; Fig. 5), while no association was detected at 6 DAS ($R^2 = 0.1$; $P = 0.4330$).

Table 2 Methylation levels measured in soybean plants treated with non-thermal plasma

Treatment	DAS	Non-methylated loci	Methylated loci
C	6	0.41 + 0.02 b	0.59 + 0.02 a
PMN3		0.42 + 0.01 b	0.58 + 0.01 a
PMO2		0.50 + 0.01 a	0.50 + 0.01 b
P-value		**0.0059**	**0.0058**
C	20	0.47 + 0.01 a	0.53 + 0.01 b
PMN3		0.39 + 0.01 b	0.61 + 0.01 a
PMO2		0.50 + 0.01 a	0.50 + 0.01 b
P-value		**0.0006**	**0.0006**

Proportion of non-methylated and methylated loci from methylation sensitive amplified polymorphism (MSAP) profiles evaluated in plants under Control (C), PMN3 and PMO2 treatments, at 6 and 20 days after sowing (DAS)

P-values in bold and different letters indicate statistically significant differences ($P \leq 0.05$)

Discussion

Non-thermal plasmas are partially ionized gases, composed of UV photons, highly reactive species of nitrogen, oxygen, and hydrogen (RNS, ROS, and RHS) among other compounds (Moreau et al. 2008; Vandamme et al. 2012; Hertwig et al. 2018). They are a novel and promising technology that might be employed for seed treatment before sowing as they allow enhancing seed health while promoting germination and vigor in a fast, cost-effective, and eco-friendly way (Jiayun et al. 2014; Panngom et al. 2014; Li et al. 2017; Hosseini et al. 2018; Yodpitak et al. 2019). It can be suggested that plasma treatments could induce oxidative stress to the exposed seeds; however, in our previous works we demonstrated that plasma treatments do not damage the seed, conversely, the highly reactive species seem to be involved in important biochemical mechanisms, functioning as molecular signals (Pérez-Pizá et al. 2018; 2019). In this sense, positive changes, concerning the antioxidant profile and phytohormone balance, were observed not only in seeds

Table 1 Non-thermal plasma-induced DNA methylation changes

	Changes at 6 DAS				Changes at 20 DAS			
Treatment	Total cases	O (%)	A (%)	Contrast 'CvsT'	Total cases	O (%)	A (%)	Contrast 'CvsT'
C	525	13.7	14.1 b		543	14.9	15.0 b	
PMN3	525	23.9	23.9 a	**< 0.0001**	543	35.3	34.9 a	**< 0.0001**
PMO2	525	25.7	26.1 a	**< 0.0001**	543	33.6	33.9 a	**< 0.0001**

Treatment effects on the probability that an epiallele changes from the consensus epigenotype based on presence/absence of 525 and 543 epialleles in three replicates of the Control plants (C) at 6 and 20 DAS, respectively. Different letters indicate statistically significant differences ($P \leq 0.05$)

O observed, *A* adjusted

P-values in bold and different letters indicate statistically significant differences ($P \leq 0.05$)

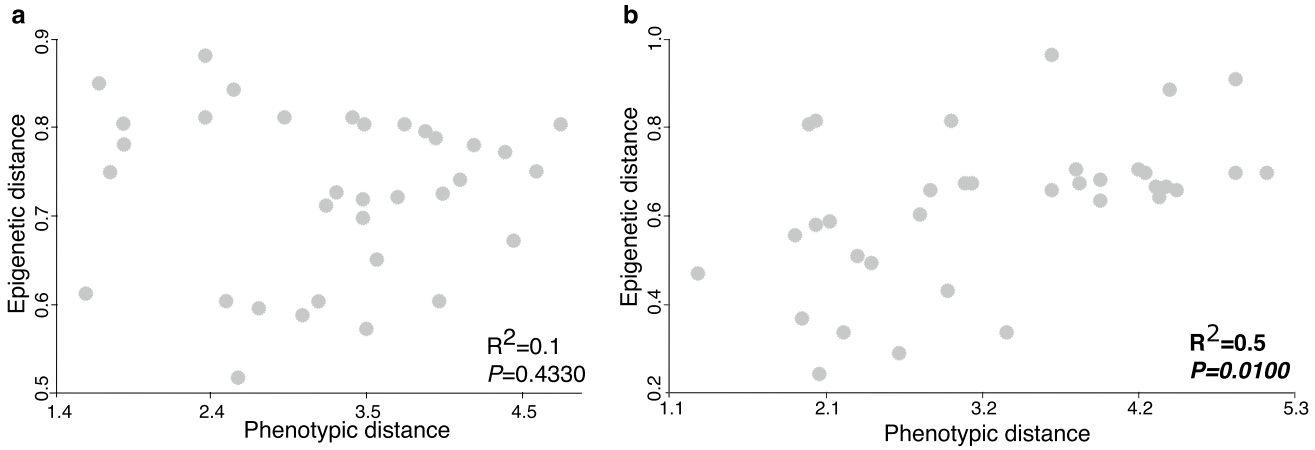

Fig. 5 Correlation between phenotypic and epigenetic variability in soybean plants treated with non-thermal plasma. Correlation based on distance matrices of epigenetic and phenotypic variability evaluated in plants under Control (C), PMN3 and PMO2 treatments, at (**a**) 6 days after sowing (DAS) and (**b**) 20 DAS

but also in the resulting plants, in response to plasma treatment. Regarding soybean, it was demonstrated that NTP can improve seed quality and produce changes in the phenotype of the resulting plants which persist after seed treatment (Ling et al. 2014; Zhang et al. 2017; Pérez-Pizá et al. 2018, 2019, 2020). In our previous researches (Pérez-Pizá et al. 2019; 2020) we evaluated the effects of different NTP treatments on seed quality, plant growth and nodulation traits. No differences were detected between PMN3 and PMO2 in this regard, despite the differences in the carrier gases.

The precise mechanisms underlying plasma effects on seeds and the plants grown from them remain unknown since multiple processes seem to be involved. Hayashi et al. (2015) proposed that the increased plant growth in response to seed treatment with plasma could be related to the enhanced production of glutathione (GSH) and polyphenols, mediated by ROS. Also, Ling et al. (2014) proposed that plasma promotes germination through enhancing enzyme digestion and, thereby, increasing the concentrations of soluble protein. According to Perrot-Rechenmann (2010), auxins promote the acidification of the extracellular matrix, triggering the action of proteins that elongate cell walls (expansins). In a previous study (Pérez-Pizá et al. 2020), we observed an enhanced plant growth (root growth in particular) in response to plasma treatment, related to higher expression of expansin genes. A possible molecular mechanism that could be associated with changes in gene expression is DNA methylation (Law et al. 2010). In the present research, our results showed that plants grown from NTP-treated seeds presented changes in DNA methylation at two different growth stages (6 and 20 DAS). About this, Zhang et al. (2017) demonstrated that argon plasma increased soluble protein and ATP concentration in soybean seeds and found a correlation between demethylation and expression of genes related to plant growth and metabolism. Liang et al. (2019) showed that, under stress conditions, the genes related to glucose catabolism, amino acid, and fatty acid anabolism were demethylated, and the DNA demethylases enhance their expression. They suggested that plants can cope with stress through demethylation. However, demethylation at 6 DAS could not be related to stress, since none of the plasma treatments provoked oxidative stress either in seeds or in seedlings (Pérez-Pizá et al. 2018; 2019). Literature affirms that during certain events of the plant cycle (e.g. germination) transient quantities of ROS are produced and employed as signal molecules in the involved metabolic processes (Considine et al. 2014; Morales and Munné-Bosch 2016). In light of this knowledge, we suggest that the observed changes in the methylation level of plants at 6 DAS could be related to transient production of ROS, inherent to the germination process.

The Control plants exhibited a demethylation trend through the phenological progress from 6 to 20 DAS. It was proposed that in soybean hypomethylation could play a more relevant role than hypermethylation in the gene expression regulation process (Song et al. 2013). It is unlikely that the observed phenotypic differences among Control and treated plants could be explained by overall changes in the methylation levels (i.e. hypo- or hypermethylation). Plants obtained from PMN3- and PMO2-treated seeds were phenotypically similar to each other at both stages of growth and differed from the Control, but PMN3 plants presented a higher proportion of methylated loci than PMO2 ones at both stages of growth. Our results indicate that both demethylation and methylation changes at specific loci are operating in response to NTP treatments.

There is evidence that shows the relation between redox intermediates and epigenetic mechanisms (Huang

et al. 2019; Saravana Kumar et al. 2020). Increases of redox intermediates could influence several pathways in plant DNA methylation: they could affect the synthesis of methyl group donors, the expression of DNA methyltransferases, and DNA demethylases, among others (for a detailed revision see Saravana Kumar et al. 2020). At 6 DAS, plants belonging to PMO2 treatment presented more non-methylated loci than Control plants while, at 20 DAS, PMN3 treatment showed a methylation level higher than Control plants. These results could indicate the differential interplay between DNA methylation and redox intermediates originated from each treatment.

In the present work, a key result is that as growth progresses, the differences between plasma treated and the Control plants are exacerbated, both at the phenotypic and epigenetic levels. The correlation results indicate that the DNA methylation patterns variability could participate in the origin of the phenotypic superiority of plants obtained from treated seeds. The non-thermal plasma treatments induced reprogramming of DNA methylation patterns, which seems not stochastic: the biological replicates arranged together and formed consistent groups. It is possible that the higher differentiation with respect to the Control plants at 20 DAS is related to a more active metabolism of plants at this stage of growth compared with 6 DAS. However, the molecular mechanisms underlying plasma effects and how plasma treatments trigger methylation changes remain unsolved.

Although MSAP has a lower genomic resolution than the approaches that combine bisulfite treatment with massive sequencing, the results of MSAP markers have been comparable to those obtained by RRBS (Reduced Representation Bisulfite Sequencing), which analyzes a greater number of epiloci in the entire genome and has a nucleotide genomic resolution (Varela et al. 2020).

In summary, our results showed that plants grown from NTP-treated seeds (PMN3 and PMO2), at 6 and 20 DAS stages of growth presented differential phenotype and DNA methylation patterns, but these parameters were only correlated at 20 DAS stage. Although at the phenotypic level we did not observe differences between the NTP treatments, at the global methylation level we found differences for each stage of growth.

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Author Contributions MCPP and VI carried out the experiments, conducted the statistical analyses, and wrote the manuscript. AV contributed to the epigenetic techniques, the analysis of the results, and the writing of the manuscript. LP, EC, MF, JCCG, and BF conceived the plasma experimental prototype and performed all the treatments. CZ and PV contributed to the analysis of the results. LP, KB, and CM conceived the research and were in charge of its overall direction and planning. All authors provided critical feedback and helped shape the research, analysis and manuscript. All authors read and approved the manuscript.

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Data Availability The datasets generated during the current study are available from the corresponding author on reasonable request.

Declarations

Conflict of interest The authors declare no conflict of interests.

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