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Plasma activated water and airborne ultrasound treatments for enhanced germination and growth of soybean

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ABSTRACT

The effect of two novel technologies, also in combination, on germination and growth of soybeans has been investigated. On one side, ultrasound treatment of the seeds increased water uptake without altering the morphology and the wettability of the seed coat, but also induced slight chemical modifications of the outer part of the seed. Plasma-activated water (PAW), obtained from treating water with non-thermal atmospheric-pressure plasma in air, increased the rate of germination and subsequent plant growth. Different combinations of these two technologies were tested in order to study their interaction and to identify an optimum treatment process. *Industrial relevance:* A great urgency in crop management is to enhance sustainability. The aim is to achieve a cheap and eco-friendly production process reducing the wide current use of energy, irrigation water, chemicals and pesticides. Soybeans is a legume whose worldwide production is increasing in the last years therefore a higher efficiency and sustainability in its cultivation is obviously very appealing. Cold plasma and Ultrasound technologies are well-known in the industrial scenario and their applications in crop production are recently drawing attention; the potential of combining these two powerful techniques is clearly very promising.

1. Introduction

Soybean (*Glycine max*) is a legume that is widely grown for its edible bean, which can be consumed without further processing, defatted to produce a proteic animal feed, treated to produce substitutes for meat and dairy products (such as tofu) and used for the production of fermented products such as soy sauce. In 2015/2016 the worldwide production of soybean was 313.71 million metric tons and an 11.10% increase has been estimated for 2016/2017 (WASDE-573). In light of land use limitations and the need for sustainable intensification of horticulture, there is a need to drive yield increases in important versatile crops such as soybean.

Cold Plasma science and technology has achieved many results since the 1970's, and it is still developing applications, processes and products (Adamovich et al., 2017; d'Agostino, Favia, Oehr, & Wertheimer, 2005). Non-thermal atmospheric-pressure plasma is becoming increasingly important in agriculture (Misra, Schluter, & Cullen,

2016; Puac, Gherardi, & Shiratani, 2018). This technique is suitable for treatment of biological samples, operating approximately at room temperature and pressure. The technology is considered environmentally friendly as systems can be designed to operate with atmospheric air only, and the process does not produce waste. Moreover, in this research field multiple applications have been explored so far, which may be classified into 3 main categories (Ito, Oh, Ohta, & Shiratani, 2018):

- 1- Decontamination of agricultural products
- 2- Enhancement of seed germination and growth of plants, beneficial microorganisms, and worms
- 3- Removal of organic volatile compounds

In particular, the second point can be mainly achieved by direct treatment of seeds (Bormashenko et al., 2015; Bormashenko, Grynyov, Bormashenko, & Drori, 2012; Dobrin, Magureanu, Mandache, & Ionita,

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2015; Filatova et al., 2014; Jiang et al., 2013, 2014; Li et al., 2014; Li, Li, Shen, Zhang, & Dong, 2015; Mitra et al., 2014; Šerá, Šerý, Štraňák, Špatenka, & Tichý, 2009; Šerá, Špatenka, Šerý, Vrchotová, & Hrušková, 2010; Stolárik et al., 2015; Zhuwen, Yanfen, Yanfen, Size, & Wei, 2011) or producing plasma activated water (PAW) used plant irrigation. Sivachandiran and Khacef (2017) studied the combined effect of non-thermal plasma (NTP) treatment of water and seeds on the rate of germination and plants growth of several species of seeds. Direct seed treatment is usually carried out in Dielectric Barrier Discharge (DBD) configuration and can enhance the seed germination by improving the wettability of its surface, increasing the antioxidative activity of cells in response to oxidative stress and other physical and chemical interactions. The production of PAW is achieved by different configurations of plasma including: DBD, gliding arcs, DC, AC or pulsed coronas. Plasma in water produces Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS) and lowers the pH, thus acting as an antibacterial agent and as a fertilizer (Lu, Bohem, Bourke, & Cullen, 2017; Oehmigen et al., 2010; Park et al., 2013). A high oxidation reduction potential (ORP) combined with a low pH is found to be responsible for the antibacterial activity of PAW. The fertilizing properties depend on the presence of two important species: H_2O_2 and NO_3^- . High levels of H_2O_2 can lead to plant cell damage due to its strong oxidizing capacities, but an appropriate amount can have positive effects on the seed germination. These include oxidation of stored proteins causing their mobilization toward the forming axis, crosstalk with other molecules and phytohormones such as abscisic acid (ABA) and gibberellins (GAs), that have a role in the dormancy/germination equilibrium, weakening of the endosperm thereby inducing programmed cell death (PCD) into the aleurone cells thus facilitating its rupture and protection against harmful pathogens (Wojtyła, Lechowska, Kubala, & Garnczarska, 2016). Moreover, nitrates are fundamental in plants nutrition given their key role in the synthesis of amino-acids, proteins, chlorophyll, etc. Compared to other fertilizers, PAW can be manufactured cheaply from water and air, which along with the absence of organic compounds and pollutants make the technique attractive.

Ultrasound, acoustic waves generated at frequencies higher than 20 kHz, are extensively used in agro-industry. The application in food industry can be divided into low energy (frequencies higher than 100 kHz at intensities below $1 \text{ W}\cdot\text{cm}^{-2}$) used for non-invasive analysis and high energy (frequencies between 20 and 500 kHz and intensities higher than $1 \text{ W}\cdot\text{cm}^{-2}$), which is disruptive and can affect physical, mechanical or chemical/biochemical properties of foods (Awad, Moharram, Shaltout, Asker, & Youssef, 2012). High energy ultrasound is commonly applied in processes, such as the destruction of foams, extraction of flavorings, filtration and drying, mixing and homogenization and many others (Sun, 2014).

In recent years ultrasound has gained greater attention as a technology to stimulate plant germination with many examples reported in literature on seeds (of different plant species) exposed to high energy ultrasound using a sonication water bath (Yang, Gao, Yang, & Chen, 2015; Wang et al., 2012; Lopez-Ribera & Vicient, 2017; Tabaru, Fumino, & Nakamura, 2015; Ghafoor, Misra, Mahadevan, & Tiwari, 2014; Miano et al., 2015). The positive effects of ultrasound treatments are due to cavitation, defined as the phenomena of the formation, growth and subsequent collapse of microbubbles, which release large magnitude of energy and induce localized extreme conditions (Wu, Guo, Teh, & Hay, 2013). This phenomenon leads to the formation of pores on the seed surface and subsequently to an acceleration of the rate of influx or uptake of water into the seed. Airborne sonication, on the other hand, is less studied and requires a very powerful and efficient power source to compensate for the greater power loss occurring during the air propagation of the waves. This technique allows the treatment of seed by modification of their coat without damage to the seed, with the dry nature of the process permitting post treatment storage. The aim of this work was to study combined effect of PAW and airborne sonication on water absorption, germination and plant growth of soybean. To the

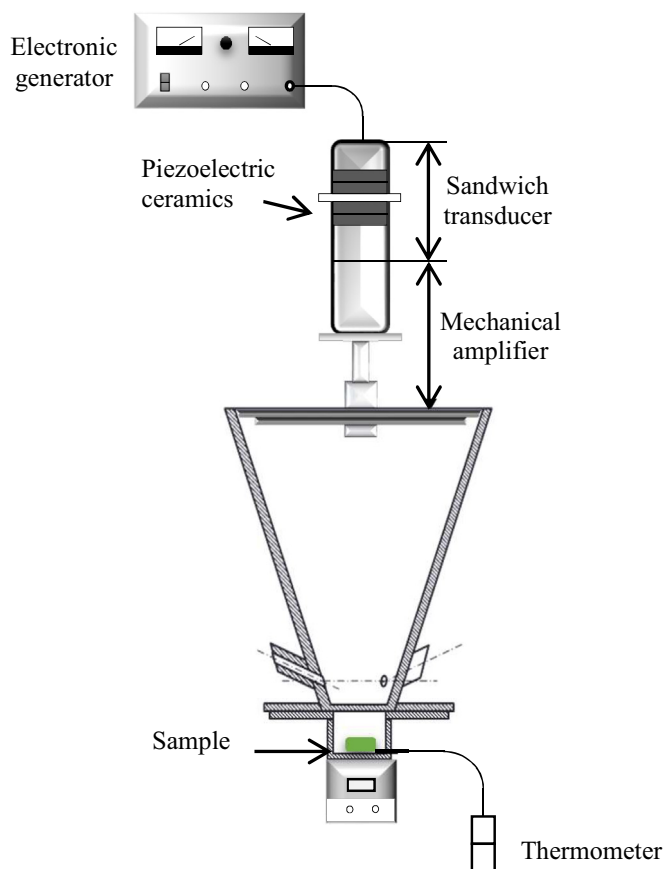


Fig. 1. Scheme of the Airborne Acoustic ultrasound generator.

best of the authors' knowledge, this is the first work reported on the combination of these two promising techniques.

2. Materials and methods

2.1. Ultrasound treatment of seeds

Soybeans (*Glycine max* (L.) Merr) were purchased from a local store. The seeds (180 g) were placed on a metallic mesh and exposed to 30 min of ultrasound treatment generated by using Airborne Acoustic, whose scheme is represented in Fig. 1, at a frequency of 25 kHz. The seeds exposed to the ultrasound treatment will be referred to as US while the non-treated seeds will be named NTS.

2.2. PAW generation

The plasma reactor used in this work was a Dielectric Barrier Discharge (DBD) system with a maximum voltage output in the range 0–120 kV_{RMS} at 50 Hz, described previously by Sarangapani et al. (2016). The apparatus presents two parallel aluminium plate electrodes of circular geometry (outer diameter of 158 mm). Two dielectric layers were placed in contact with the high voltage electrode and the ground electrode with a thickness respectively of 10 mm and 2 mm. The plasma discharge was generated at atmospheric air, 80 kV voltage and a frequency of 50 Hz in alternating current (AC). For generation of PAW, prior to each treatment, a Petri dish containing 20 mL of deionized water was confined into a polypropylene container (310 mm \times 230 mm \times 22 mm) and sealed inside a high barrier polypropylene bag (Cryovac, B2630, USA), to avoid dispersion of volatile chemical species produced during the discharge. The package containing the samples was placed directly in contact with the dielectric layers, in the plasma zone, and treated for either 1 min or 5 min (P1 and

P5). The chemical characterization of plasma activated water using this device has been previously reported by [Boehm, Curtin, Cullen, and Bourke \(2017\)](#).

2.3. Characterization of PAW

The chemical characterization of water was carried out by measuring pH, NO_2^- , NO_3^- and H_2O_2 . pH was acquired by means of a pH-meter. Nitrite concentration was measured spectrophotometrically (540 nm) following the reaction with Griess reagent. Nitrate concentration was measured using a Merck Nitrate assay kit (Spectroquant nitrate test 1.09713.0001) based on the photometrical determination (340 nm) of 4-nitro-2,6-dimethylphenol formed following the reaction of ions with 2,6-dimethylphenol (DMP). The method used for the detection of H_2O_2 was based on the oxidation of iodide (I^-) in yellow iodine which can be measured spectrophotometrically (390 nm). The quantification of the analytes is achieved through comparison with a calibration curve.

2.4. Chemical and morphological characterization of seeds

2.4.1. ATR-FTIR absorption spectroscopy

ATR-FTIR was carried out on NTS and US treated seeds to identify any chemical changes induced on the seed surface after the ultrasound treatment. ATR spectra (32 scans per analysis, 4 cm^{-1} resolution) were obtained in transmission mode with a Vertex 70 V Bruker spectrometer. The spectrometer was evacuated to less than 150 Pa for 10 min before each acquisition. Spectra were normalized by the maximum intensity of the CH_x stretching band at 2935 cm^{-1} .

2.4.2. Water contact angle measurement

Water contact angle (WCA) was acquired by means of a contact angle meter (Theta Lite Optical Tensiometer, Biolin Scientific, UK) and Attension software. The images were recorded at 15 frames per second for 10 s. The drop volume chosen was $9\text{ }\mu\text{L}$. The WCA for each sample corresponds to the average value obtained for 5 different seeds ($n = 5$).

2.4.3. Scanning Electron Microscopy investigation

Scanning Electron Microscopy (SEM) analysis were carried out with a Zeiss Supra 40 equipped with a Gemini field-effect emission gun at an extraction voltage of 5 kV. The analyses were carried on the coats of NTS and US, upon metallization with 30 nm thick layer of chromium, by means of a Quorum Q150 sputter coater. The coats were detached from the seed by placing them under vacuum (10–5 Torr). For each acquisition the brightness, the contrast and the working distance (varying in the range of 2–4 mm) were optimized.

2.4.4. Fluorescence Microscopy

To observe differences in water uptake following ultrasound exposure the NTS and US seeds were dipped for 5 min in a fluorescein solution ($100\text{ }\mu\text{g/mL}$) and then imaged with a Zeiss Aimat (Germany) epifluorescence microscope.

2.4.5. Seed water uptake

Water absorption of both NTS and US was evaluated after immersion in water (W), P1 or P5. Different immersion times were chosen: 15, 30, 45, 60 min. For each sample 5 seeds were placed in a tube immersed in 4 mL of liquid and the measure was obtained in triplicate ($n = 3$). After immersion the seeds were extracted from the tubes, gently blotted and left to dry for 2 h. The percentage of absorbed water (W_a) per sample was calculated according to Eq. (1), where W_i is the initial weight and W_f is the final weight of seeds, and an average value was recorded.

$$W_a\% = \frac{(W_i - W_f)}{W_i} \cdot 100 \quad (1)$$

2.5. Seed germination and growth characterization

Seed germination was evaluated over 3 days as this is the time required for full germination. For each sample 9 Petri dishes containing a layer of filter paper on the bottom and 10 seeds each, were prepared. Each Petri dish was watered with 2 mL of W (control water), P1 or P5 every day. A seed was considered “germinated” when a 2 mm long sprout was visible. The germination percentage was calculated for each Petri dish according to Eq. (2) and then an average was calculated over all Petri dishes available for each sample.

$$G\% = \frac{n^\circ \text{ of germinated seeds in each Petri dish}}{\text{total } n^\circ \text{ of seeds in each Petri dish}} \cdot 100 \quad (2)$$

Plant growth was assessed as follows: for each sample 6 pots were prepared containing 30 g of soil and 1 seed. The seeds were placed approximately 1.5 cm underneath the surface of the soil and at the center of the pot. Each pot was watered with 5 mL of water every day, except on Friday when 10 mL were added to compensate for the weekend. The length of each stem was measured every day and an average value was calculated over the 6 pots ($n = 6$). The temperature and the light exposure during the experiment were ambient temperature and natural light and the position of the pots was varied every day in order to minimize the effect of the light. The experiment lasted 20 days (06/06/2017–26/06/2017).

After 20 days of the soil growth experiment, each plant was extracted from the soil, washed and gently blotted. The roots were removed, and the remaining parts were weighed (Fresh Weight W_f), then dried in an oven overnight at $102\text{ }^\circ\text{C}$ and weighed again (Dry Weight W_d). The moisture content (MC%) was calculated using Eq. (3).

$$\text{MC}\% = \frac{(W_f - W_d)}{W_f} \cdot 100 \quad (3)$$

3. Results and discussion

3.1. Characterization of plasma-activated water

In agreement to previous studies, also in this work air plasma treatment of water led to the formation of RONS species and to the decrease of pH. [Table 1](#) reports the concentration of nitrate/nitrite and H_2O_2 generated in water with 1 and 5 min long plasma treatment. Longer treatment time resulted in higher concentrations of NO_3^- and H_2O_2 , however, NO_2^- was not detected. The influence of plasma treatment on water pH is depicted in [Fig. 2](#). Plasma treatment drastically reduced water pH in 1 min from 4.9 ± 0.2 (W) to 3.5 ± 0.2 (P1), while for longer treatment time pH decreased at a slower rate reaching a value of 2.7 ± 0.1 (P5).

3.2. Chemical and morphological characterization of ultrasonicated seeds

Water absorption analysis, reported in [Fig. 3](#), showed that all US samples absorbed more water than the non-treated control seeds. It can be supposed that the interaction between the ultrasound waves and the surface of the seeds is responsible for a higher and easier absorption of water as a result of chemical or morphological modifications. On the other hand, the impact of the water treatment time on the water

Table 1
 NO_2^- , NO_3^- and H_2O_2 concentrations (μM) in samples of W, P1 and P5. ND is for non detected.

	W	P1	P5
NO_2^-	ND	ND	ND
NO_3^-	ND	20 ± 1	170 ± 9
H_2O_2	ND	20 ± 1	100 ± 5

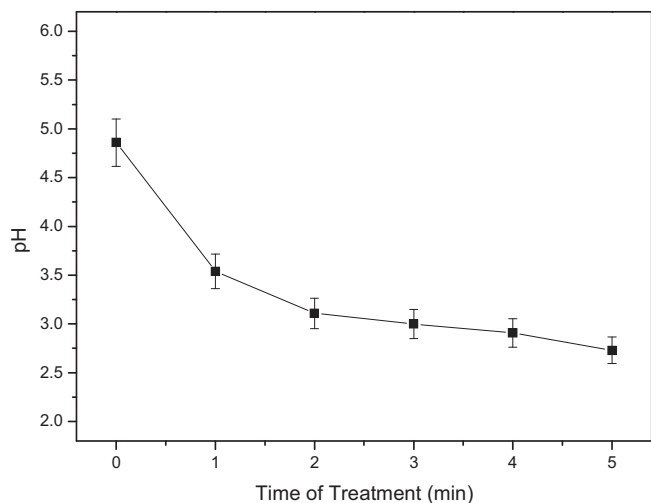


Fig. 2. Water pH as a function of plasma treatment time. Vertical bars represent standard deviations.

absorption seems to be irrelevant.

To better identify the changes induced by the ultrasound treatment, the wettability has been investigated in terms of WCA and data are presented in Table 2. It can be observed that airborne ultrasound does not influence hydrophobic properties of the seed coat as no significant difference in WCA values were found for NTS and US using as probing liquid W, P1 and P5. These results suggest that the higher water absorption of the US, compared to the NTS, is not due to an increase in the wettability of the seed coat.

On the other hand, as it can be observed in Fig. 4, though FTIR spectra are quite complex, the absorption intensity of the bands at 1318 cm⁻¹ and 1022 cm⁻¹ clearly decreases upon the ultrasound treatment. It is not easy to attribute these bands to specific chemical bonds, but it is undeniable that a chemical change occurred on the surface of the seed as a result of the contact with ultrasound waves.

SEM analysis, reported in Fig. 5, was carried out to study the morphology of the seed coat subjected to 30 min of ultrasound treatment, which demonstrated that seeds were not affected by the treatment.

The formation of pores of nanometric dimensions cannot be excluded given the high complexity of the coat surface and the presence of the metallic coating that could mask them.

Table 2
Static WCA values (°) of the seeds (NTS or US) using W, P1 or P5.

	W	P1	P5
NTS	109 ± 4	106 ± 6	105 ± 5
US	107 ± 6	107 ± 6	110 ± 4

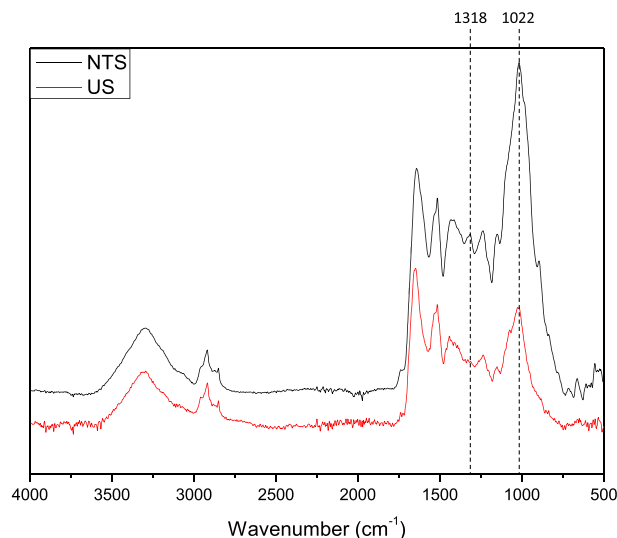


Fig. 4. ATR spectra of NTS and US.

Since the chemical-morphological results reported so far do not led to well identify the reason for the increased water absorption of US, fluorescence microscopy was used to better characterize such absorption. Seeds were immersed for 5 min in fluorescein solution and their sections were observed at the microscope. The results are reported in Fig. 6 and indicate a different path of water absorption for NTS and US. In particular, NTS seeds show fluorescence originating almost completely from the coat, with a faint emission from the abaxial parenchyma, suggesting a superficial uptake. The seed section images for US, instead, show that fluorescence originates from the inner part of the cotyledon, in particular from the vascular bundles. Vascular bundles fulfill a very important role in the transport system of the plant, allowing transportation of water, nutrients and signals. Therefore, the fluorescence location is consistent with a deeper, and faster, water

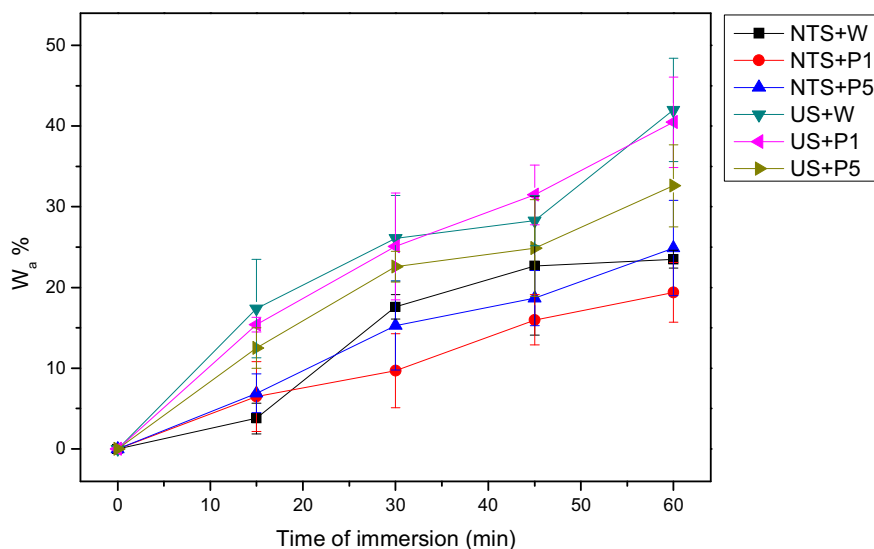


Fig. 3. Values of W_a % vs the time of immersion of the seeds (NTS or US) in water (W, P1 or P5). Vertical bars represent standard deviations.

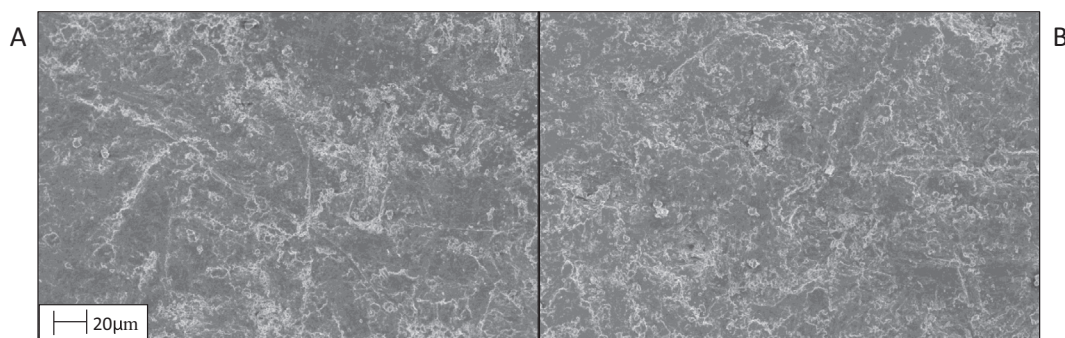


Fig. 5. SEM images of the coat of the seeds A) NTS and B) US.

uptake in the US samples. This result confirms the higher water absorption measured and reported above for seeds exposed to ultrasound waves (Fig. 3).

3.3. Seed germination and growth characterization

The germination percentage (G%) of either NTS or US watered with either water (W), plasma-treated water for 1 or 5 min (P1 or P5, respectively) was recorded every 24 h for 3 days. The same G% was obtained for all samples on Day 1 (0%) and on Day 3 (100%). However, a noticeable difference between G% for different samples was observed on Day 2, with higher germination obtained for US watered with plasma treated water and results are reported in Fig. 7. In the case of NTS, higher germination was obtained for seeds treated with P5 as compared with W. Hence, in general watering with plasma treated water is effective in enhancing seed germination for untreated seeds, whereas it is not so relevant when the seeds are ultrasonicated. On the other hand, the ultrasound treatment can positively influence the formation of sprouts.

Fig. 8 shows that the optimum watering option for the growth of NTS is to use P1 while P5, even if more effective than W, seems to be slightly less active. Using P1 is the better option also for watering US but P5 in this case does not differ from W. P5 is more acid so the interaction with the soil can have negative effects on plant growth. This could be due to the leaching of poisonous metals such as aluminium, cadmium and mercury from the soil in which they are normally bonded; aluminium, in particular, is toxic to plant roots in its free organic form as it can lock up phosphate (an important plant nutrient) thus reducing its concentration available in the soil. Moreover, increasing the soil acidity can affect benign micro-organisms which have a role in providing nutrients from soil by breaking down organic matter. The negative effect of the acidity of P5 is more evident on the US probably because the higher permeability of their coat makes them more exposed to the surrounding environment and, consequently, more

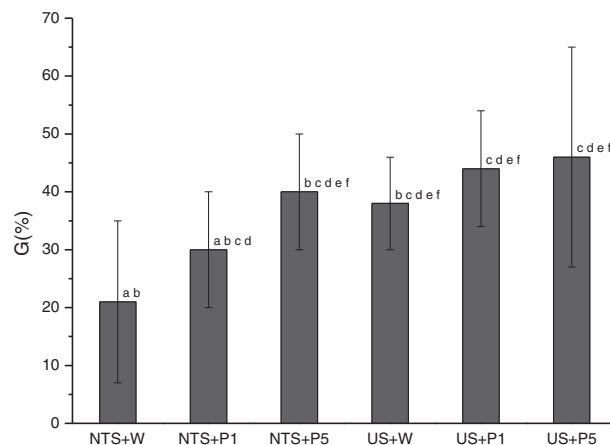


Fig. 7. Values of G % for NTS and US watered with W, P1 and P5 obtained on Day 2. Statistical analyses conducted with Student's *t*-test (level of confidence of 95%).

susceptible to the drawbacks of the acidification of the soil. Both at Day 10 and at Day 20 the seeds (NTS and US) watered with P1 appear to grow faster, to be healthier and to have a higher germination ratio as illustrated in Fig. 9.

The plants grown after 20 days were recovered and moisture content evaluated. The results are reported in Fig. 10 and it can be observed that it is very similar for the tested conditions. However, plants grown from US watered with PAW have a moisture content statistically higher than the control (NTS + W) and this means a better hydration of the plants. The trend seems to follow that of the germination ratio using the petri dishes.

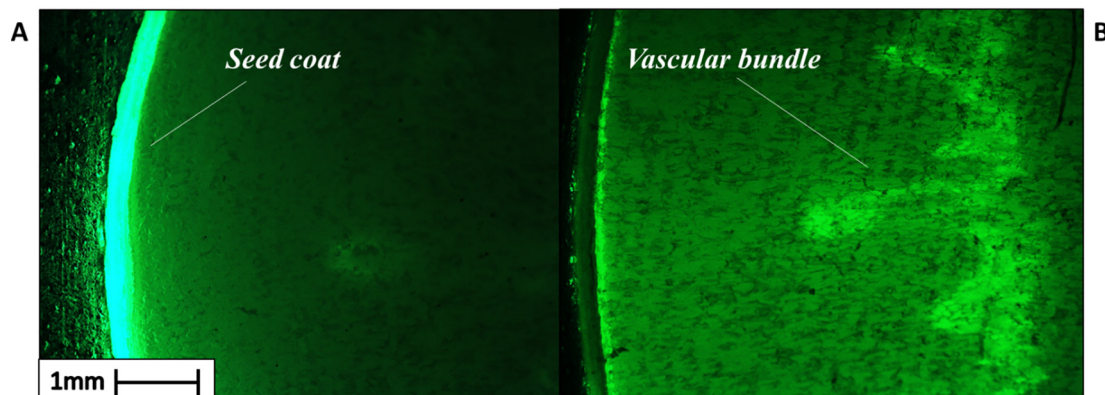


Fig. 6. Fluorescence microscopy images of seed section for a NTS (A) and a US (B).

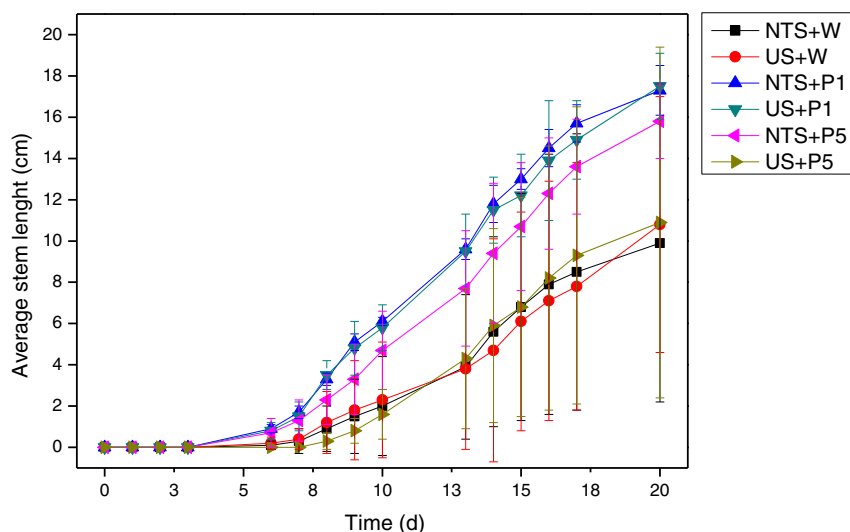


Fig. 8. Values of Average stem length vs the time of germination of the seeds (NTS or US) daily watered with W, P1 or P5). Vertical bars represent standard deviation.



Fig. 9. Images of the plants growing from the seeds (NTS or US) daily watered with W, P1 or P5). A) 10 days after sowing, from top view; B) 20 days after sowing, from top view; C) 10 days after sowing, from side view; D) 20 days after sowing from side view.

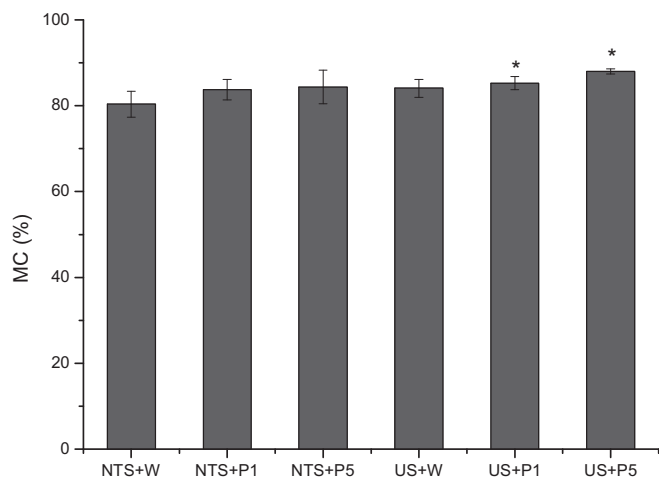


Fig. 10. Values of Moisture Content (MC%) on Day 20 of germination of the plants from the seeds (NTS or US) daily watered with W, P1 or P5). Vertical bars represent standard deviation. Star (*) represents samples statistically different from the control (NTS + W) (Student's *t*-test, level of confidence of 95%).

4. Conclusions

The ultrasound treatment induces chemical modifications on the seed surface of soybean (observed with ATR) and, although morphological changes were not observed, the formation of pores of nanometric dimensions are not excluded (showed by SEM images). These modifications do not affect the hydrophobicity of the coat, as proved by WCA analyses, but cause a higher permeability to water resulting in a higher water absorption of the seeds exposed to the ultrasound treatment (US). From the fluorescence microscopy images it could be inferred that, the water absorption is superficial for the non-treated seeds (NTS) however conversely for the US sample the fluorescein solution permeated through the coat to the vascular bundles.

Using germination time and it appears that longer plasma treatment times for plasma activated water yields a chemical cocktail that results in faster germination.

In soil the situation was different, with the best growth results obtained with P1 for both US and NTS. No difference was observed between untreated and ultrasound treated seeds watered with distilled water, but a considerable difference was noted between when P5 water was used. In the soil the interaction between the water and soil has an impact on plant growth with P1 having sufficient quantities of oxidizing species necessary to stimulate seed growth more than W. P1 was also

not too acidic to negatively impact seed performance. P5 on the other hand had a very low pH, which can negatively affect soil generating harmful species and potentially limiting the plants growth. This was especially true for the US, which were more exposed to this harsh environment due to the higher permeability of their surface.

The moisture content measurement demonstrates the higher plant hydration achieved for ultrasound treated seeds, in agreement with the higher absorption rate found.

In conclusion, exposing soybean seeds to airborne ultrasound waves can increase the hydration of the plants and the ability of the seeds to absorb water. When water which was plasma-treated using the in-package DIT-120 system for 1 min (P1) was used for watering the seeds in soil, faster growth and taller soybeans plants are observed. The impact and interaction of ultrasound waves and plasma treatment of water was studied, which point to an optimum condition between these novel technologies.

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