

Plasma applications for the treatment of bean sprouts: safety, quality and nutritional assessments under aqueous and gaseous set-ups

Martina Darmanin

University of Malta

Antje Fröhling

Leibniz Institute for Agricultural Engineering and Bioeconomy

Sara Bußler

Leibniz Institute for Agricultural Engineering and Bioeconomy

Julia Durek

Leibniz Institute for Agricultural Engineering and Bioeconomy

Susanne Neugart

University of Göttingen

Monika Schreiner

Leibniz Institute of Vegetable and Ornamental Crops

Renald Blundell

University of Malta

Ruben Gatt

University of Malta

Oliver Schlüter (✉ oschlueter@atb-potsdam.de)

Leibniz Institute for Agricultural Engineering and Bioeconomy

Vasilis P. Valdramidis

University of Malta

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Abstract

Sprouts are particularly prone to microbial contamination due to their high nutrient content and the warm temperatures and humid conditions needed for their production. Therefore, disinfection is a crucial step in food processing as a means of preventing the transmission of bacterial, parasitic and viral pathogens. In this study, a dielectric coplanar surface barrier discharge (DCSBD) system was used for the application of cold atmospheric plasma (CAP), plasma activated water (PAW) and their combination on mung bean seeds. Overall, it was found that the combined seed treatment with direct air CAP (350 W) and air PAW had no negative impact on mung bean seed germination and growth, nor the concentration of secondary metabolites within the sprouts. These treatments also reduced the total microbial population in sprouts by 2.5 log CFU/g. This research reports for first time that aside from the stimulatory effect of plasma discharge on seed surface disinfection, sustained plasma treatment through irrigation of treated seeds with PAW can significantly enhance seedling growth. The positive outcome and further applications of different forms, of plasma i.e., gaseous and aqueous, in the agro-food industry is further supported by this research.

Introduction

Cold plasma technology is leading major breakthroughs in addressing a plethora of issues in the agriculture and food sectors, from mitigating produce losses due to pathogens and pests, to enhancing the yields and safety of food (Bourke et al., 2018, Knorr et al., 2011, Puač et al., 2018, Bußler, 2017). Being an ionised gas, cold atmospheric plasma (CAP) constitutes of a complex mixture of active agents, such as UV photons, charged particles, radicals and other reactive nitrogen, oxygen and hydrogen species (RNS, ROS and RHS) (Lu and Wu, 2013, Thirumdas et al., 2018). These reactive species, individually and/or synergistically, demonstrate microbial inactivation properties while also play a role in promoting germination, rooting and growth of plants (Adamovich et al., 2017, Darmanin et al., 2020). Cold plasma processes are most efficient and have the least negative impact on food at atmospheric pressure and low temperatures (generally < 70°C) (Schluter et.al, 2013).

A plasma system, including its electrode configurations and its process parameters concerning energy supply, working gas and treatment time all affect the composition of the generated plasma and consequently also the antimicrobial and germination efficiency of the plasma treatment (Lukes et al., 2012). The versatility of plasma set-ups enable unique designs for the precise generation of plasmas and controlled production of acting agents compatible with the needs of the agro-food industry (Schluter, et.al, 2013). CAP treatment, having a low impact on the internal product matrix and being overall a resource-efficient application free of water (in case of CAP only), solvents and residues, is considered a potential alternative to conventional chemical disinfection treatments and physical disinfection methods, such as thermal and high-pressure treatment, pulsed electric fields, and ionizing irradiation (Pankaj et al., 2018, Lung et al., 2015).

The growing popularity of sprout consumption may be attributed to research from nutritional experts revealing the chemistry of sprouting seeds and their biological value in human and animal nutrition (Marton et al., 2010, Laila and Murtaza, 2014). Apart from being a significant source of amino acids, proteins, fibre, enzymes, vitamins and minerals fundamental to human health (Marton et al., 2010), sprouts also naturally contain a number of bioactive compounds, known as phytochemicals (Zlotek et al., 2015). The principal phytochemicals found in sprouts of the legume family, including alfalfa, mung bean, clover, peas, chickpeas and soybeans are phenolic compounds (Zlotek et al., 2015). Epidemiological data identified an inverse relationship between the intake of phenolic rich food and the rate of chronic diseases such as diabetes, cardiovascular diseases, Alzheimer's disease, Parkinson's disease and inflammation (Mohamed, 2014). Flavonoids are the most common subtype of phenolic compounds and have the potential to alter lipid metabolism, reduce atherosclerotic lesion formation, improve endothelial function, prevent platelet aggregation and reduce blood pressure (Vauzour et al., 2010). Flavonoid-containing sprouts of cash crops with specific health benefits include buckwheat sprouts, mung bean sprouts, barley sprouts and fenugreek sprouts (Meghwal, 2012, Kim et al., 2012, Nam et al., 2015, Lee et al., 2016). Flavonoids have a number of protective roles, primarily on vulnerable neurons with a simultaneous ability of

stimulating neuronal regeneration in order to maintain and enhance brain function (Vauzour et al., 2010). Furthermore, flavonoids have the ability to quench free radicals to prevent oxidative rancidity, a major cause of food quality deterioration (Embuscado, 2015). Chemoprevention is another notable benefit of flavonoids (Link et al., 2010, Vauzour et al., 2010).

Disinfection is a crucial step in food processing as a means of preventing the transmission of bacterial, parasitic and viral pathogens and outbreak of food-borne illness from contaminated fresh produce (Ziuzina, et al., 2014). Sprouts are particularly prone to contamination due to their high nutrient content and the warm temperatures and humid conditions needed for their production (Yang et al., 2013). As sprouts are also often consumed raw, disinfection of seeds is obligatory according to the European Sprouted Seeds Association ((2017/C 220/03) in order to ensure that this healthy, inexpensive, convenient food is, above all, consumer safe.

Few studies have assessed, in parallel, cold atmospheric plasma's properties on microbial inactivation, seed germination and composition of secondary metabolites. Among the different plasma sources cited in the literature, the dielectric barrier discharge (DBD) gains attention as it allows for homogeneous CAP treatment of large volumes that is ideal for seed treatment (Sivachandiran and Khacef, 2017). Furthermore, the use of air as a process gas is a common trend for cold plasma application on sprouts, notably due to the presence of O₂ and N₂, the parent molecules of reactive oxygen and nitrogen species (RONS) that function as the prime antimicrobial agents (Xiang et al., 2019), with an additional impact on enhancing seed germination (Kitazaki et al., 2014, Zhou et al., 2019). Apart from the direct treatment of seeds with CAP, a significant branch of cold plasma research is being developed in the realm of plasma activated water (PAW). The latter is the product of CAP reacting with water, whereby the ionised gas reacts within or with the surface of water to create reactive species in the water related to the properties of CAP (Thirumdas, et al., 2018, Surowsky et al., 2016).

Further efforts at standardising cold plasma technology on food are required in order to determine if cold plasma technology complies with the European Commission's 'Novel Food' regulation (EU) 2015/2283. This should involve research evaluating the safety and quality parameters (i.e., nutrient content, colour, texture and chemical composition) of cold plasma treated food (Bovi et al., 2019). In this study, a coplanar surface barrier discharge system was used for the application of CAP and PAW treatment on mung bean seeds. The efficacy of the combined application of CAP and PAW on the germination and growth of mung bean seeds was assessed and the antimicrobial functionality of the PAW was also evaluated in order to report its disinfectant capacity. Additional analysis was carried out on the decontamination efficiency of combined CAP and PAW treatment on the natural microbiota within mung bean sprouts. Ancillary analyses were carried out in order to characterise the reactive species in the plasma activated water and to assess the composition of secondary metabolites in the harvested sprouts.

Results

Seed Germination and Growth

Individual application of CAP and PAW on mung bean seeds

Preceding the combined CAP and PAW treatment of mung bean seeds, experimental assessment was performed on the CAP and PAW treatments individually. The percentage of mung bean seeds germinated within 96 h, illustrated in Fig. 1, was 97.5% for the control, CAP (air), CAP (N₂), PAW (air), PAW (N₂) and PAW (CO₂) treatments and 90% for CAP (CO₂) treatment. As observed in Fig. 2, mung bean sprouts grown from CAP (air) and CAP (N₂) treated seeds showed a very slight increase in average stem length of 40 mung bean seed replicates compared to the average stem length of the control and CAP (CO₂) over 96 h germination. As seen in Fig. 3, the three PAW treatments demonstrated similar mung bean stem lengths compared to the control, over 96 h of growth. The growth rate of the sprout stems, expressed as rate constant (k) values (1/h) and doubling time (Dt), i.e., the time (h) taken for the sprouts to double in length were obtained using the exponential growth equation and reported in Table 1.

Table 1

Growth rate (k) and doubling time (Dt) of mung bean sprouts grown from CAP treated seeds and PAW irrigated seeds.

	Control (CAP)	CAP (air)	CAP (N ₂)	CAP (CO ₂)	Control (PAW)	PAW (air)	PAW (N ₂)	PAW (CO ₂)
k	0.037	0.037	0.034	0.031	0.038	0.034	0.036	0.036
Dt (h)	18.65	18.73	20.66	22.69	18.06	20.13	19.21	19.33
R ²	0.9909	0.9817	0.9822	0.9922	0.9958	0.9935	0.9953	0.9957

As observed in Table 1, CAP (air) appeared to be the most comparable to the control from other CAP treatments while also seemed to fair better from all plasma (CAP/PAW) treatments by demonstrating the highest growth rate of sprout stem (0.037 1/h) and fastest doubling time (18.73 h). Based on this assessment CAP (air) treatment of seeds was selected for the combined plasma treatment assessments.

Combined CAP and PAW treatment of seeds

Figure 4a–d illustrates the averaged stem length of 20 mung bean sprouts for each of the three sample replicates, recorded at various time intervals over 96 h of growth. Throughout the 96 h experiment seeds were grown on a bed of glass beads, irrigated with 3 mL of PAW contained in test tubes and kept incubated in the dark at 25°C ± 1°C at 45% humidity. The growth rate and doubling time were obtained using the exponential growth equation and reported in Table 2.

Table 2

Growth rate constant (k) and doubling time (Dt) of mung bean sprouts of sample replicates 1–3 for each combined plasma treatment obtained from exponential growth equation. Goodness of fit represented by R squared (R^2) value. Profile likelihood of each value demonstrated using 95% Confidence Interval (CI).

	Control		CAP + PAW (air)		CAP + PAW (N ₂)		CAP + PAW (CO ₂)					
	CI _{upper}	CI _{lower}	CI _{upper}	CI _{lower}	CI _{upper}	CI _{lower}	CI _{upper}	CI _{lower}				
k_{1-3} (1/h)	0.033	0.035	0.031	0.033	0.034	0.031	0.034	0.036	0.031	0.032	0.034	0.030
Dt_{1-3} (h)	20.77	22.09	19.57	21.28	22.50	20.16	20.67	22.12	19.35	21.70	23.24	20.31
R ²	0.9709		0.9864		0.9800		0.9793					
RMSE	0.2877		0.2140		0.2618		0.2287					

As observed in Table 2, the growth rate of mung bean sprouts appeared to be relatively similar with only a 0.001 1/h difference between the control and the combined plasma treatments in N₂ [CAP + PAW (N₂)] and in CO₂ [CAP + PAW (CO₂)]. In comparison to the control, the Dt varied by + 0.77 h, -0.10 h and + 0.93 h for the combined plasma treatments in air [CAP + PAW (air)], N₂ and CO₂ respectively. However, statistical analysis determined no significant difference between the k and Dt values of the control to the treated groups as the p values (P) exceeded the 0.05 minimum level of significance in each case.

Mung bean seed germination percentage, i.e., the percentage of seeds that sprouted by the end of the 96 h experiment under the different treatments are presented in Fig. 5. While slight increases in germination percentage were demonstrated by the combined plasma treatments in air (+ 4.3%), in N₂ (+ 6%) and in CO₂ (+ 6%) compared to the control, these increases were not found to be significantly different ($P > 0.05$).

Figure 6a-d demonstrate the distribution of sprouts according to the stage of growth, in terms of stem length (cm) achieved by the end point of the experiment, i.e., 96 h. Stage of growth is represented by 5 categories of stem length (cm) on the x-axis (0.1 cm - 2.0 cm, 2.1 cm - 4.0 cm, 4.1 cm - 6.0 cm, 6.1 cm - 8.0 cm and 8.1 cm - 10.0 cm). A Gaussian curve was fitted to give an overview of the performance of the sprouts in relation to the combined plasma treatment applied and to compare frequency distributions between treatments (Fig. 6). In contrast to the 39% of sprouts from the control group that achieved an end-point stem length between 6.1 cm and 8.0 cm, 60%, 58.5% and 31.5% of sprouts from the combined plasma treatment groups in air, N₂ and CO₂, respectively grew within the same category within 96 h. However, when using statistical analysis to compare the frequencies at the respective growth stages of the control to those of the combined plasma treatments, no significant difference (P > 0.05) was determined between either of the growth stages of each treatment. For the 6.1-8.0 cm stem length category, although the combined plasma treatments in air and N₂ (as seen in Fig. 6) appeared to have a higher frequency than the control, no significant difference was observed with a p-value of 0.1040 and 0.1400, respectively.

Indication Of CAP And PAW Processing

The averaged pH of the PAW samples produced throughout the study were 3.34 ± 0.07 , 3.91 ± 0.05 and 3.64 ± 0.09 for PAW (air), PAW (N₂) and PAW (CO₂), respectively. The temperature of the PAWs was monitored after treatment and was never found to exceed room temperature.

Characterisation Of The Irrigation Plasma Activated Water

Following irrigation of seeds with PAW, the irrigation water was collected after 48 h and 96 h of germination. The water samples were pooled and the concentration of nitrite (NO²⁻) and nitrate (NO³⁻) species in PAW samples were determined using an ICS-1000 ion chromatography system (Thermo Scientific Dionex, Germany), with a sample injection volume of 25 µl. The anions were eluted using a 4 × 250 mm IonPac® AS9-HC anion-exchange column (Thermo Fisher DIONEX, Germany), equipped with an ASRS-Ultra detector. The eluent was Na₂CO₃ (9mmol) and the flow rate was 1.2 ml/min.

Table 3.

Concentration (mg/L) of nitrates and nitrites in the irrigation PAWs collected after 48 h and 96 h of seed germination.

	Nitrate concentration (mg/L)		Nitrite concentration (mg/L)	
	48 h	96 h	48 h	96 h
Control	0.12 ± 0.02	0.04 ± 0.01	<0.02	<0.02
PAW (air)	2.72 ± 0.27	2.10 ± 0.30	2.69 ± 0.71	1.26 ± 0.80
PAW (N₂)	0.44 ± 0.06	0.12 ± 0.05	0.82 ± 0.13	0.16 ± 0.11
PAW (CO₂)	2.72 ± 0.54	1.70 ± 0.25	0.02 ± 0.02	0.01 ± 0.02

Antimicrobial Capacity Of PAW

The impact of PAW on *E. coli* (log CFU/mL) is depicted on Fig. 7. Evidently, a microbial reduction that went below the detection limit (1.3 log CFU/mL) was achieved within 4 h while the population of *E. coli* when exposed to the control, PAW (N₂) and PAW (CO₂) was steady and between 7 and 8 log CFU/mL. The kinetic data were analysed with an exponential decay equation from which the inactivation rate, expressed as rate constant (*k*) values (1/h) of *E. coli* exposed to PAW (air) and half-life (*H*) was determined (refer to Table 4). Figure 8 illustrates the total bacterial reduction of *E. coli* (log CFU/mL) within 6 h of exposure to each treatment. Significant reduction (P = 0.0013) in the population of *E. coli* exposed to PAW (air) (CI_{upper}: -7.046 log CFU/mL; CI_{lower}: -7.501 log CFU/mL) was found when compared to the control.

Table 4
 Inactivation rate constant (k) and half-life (Hl) of *E. coli* exposed to PAW (air) replicates 1–3 obtained from the exponential decay equation. Goodness of fit represented by R squared (R^2) value. Profile likelihood of each value demonstrated using 95% Confidence Interval (CI).

PAW (air)			
		CI _{upper}	CI _{lower}
k_{1-3} (1/h)	0.60	0.9262	0.3408
Hl_{1-3} (h)	1.16	2.034	0.7484
R^2	0.913		
RMSE	0.8475		

**P ≤ 0.01

Decontamination efficiency of CAP and PAW treated sprouts

The starting microbial population in the seeds was 2.79 ± 0.33 log CFU/g and 2.72 ± 0.60 log CFU/g for the control and CAP treated seeds, respectively. The microbial population (log CFU/g) of the combined plasma treated sprouts recorded on day 2, 3 and 4 of the sprout growth is depicted in Fig. 9. Sprouts of the CAP and PAW (air) treatment group maintained the best control over microbial proliferation throughout the 4 days of growth, measured at 4.92 log CFU/g on day 4, compared to the control sprouts (7.46 log CFU/g) and combined plasma treatments in N_2 and CO_2 (6.78 and 6.90 log CFU/g respectively). In fact, on day 4, the microbial population within the sprouts of the combined plasma treatments in air ($P = 0.0053$) and CO_2 ($P = 0.0092$) were found to be significantly lower than that of the control. The variation between sample replicates may be attributed to biological variation between the sprouts and may have been reduced with further process replications.

Analysis Of Secondary Metabolites

The concentration of different flavonoids in the sprouts is depicted in Fig. 10, where results are presented as mg/g dry weight. Overall, the concentration of secondary metabolites is relatively similar between sprouts from the control and the different combined plasma treatments with no significant disparities.

Discussion

The interactions between the plasma generated RONS and water molecules have been found to enrich water with biochemically active species that demonstrate significant disinfection (Lukes et al., 2014, Ikawa et al., 2016, Zhou et al., 2018) and seed germination enhancing properties (Sivachandiran and Khacef, 2017, Sarinont et al., 2017, Zhang et al., 2017), over a longer exposure time than the RONS generated during direct CAP treatment. The latter treatment plays a significant role in the surface modification of the seed coat, by increasing its surface wettability and increasing the absorption of water into the seed (Xiang et al., 2019). The acidic environment generated in PAW (air) ($pH 3.34 \pm 0.07$) correlates with the higher levels of NO_3^- species quantified in PAW (air), as HNO_3 is one of the leading agents causing acidification of PAW. Relatively higher pH values were observed in PAW (N_2) (3.91 ± 0.05) and PAW (CO_2) (3.64 ± 0.09) as the quantity of reactive nitrogen species decreased. Further analysis on the physicochemical properties of the PAWs produced include the quantification of hydrogen peroxide, that is another long lived reactive species with important

functions in microbial deactivation and seed germination, and the characterisation of PAW exactly after its generation in order to compare the starting quantity of RONS with other literature.

Similar to the germination assessment outcomes of individual CAP and PAW treated mung bean seeds, the three combined CAP and PAW treatments showed no significant stimulatory effect on mung bean seed germination or performance, although as relevant is the lack of inhibitory effect. This outcome contradicts the body of research evidencing significant increases in germination rate following cold atmospheric pressure plasma treatment of seeds (Sera et al., 2010, Mitra et al., 2013, Tong et al., 2014, Meng et al., 2017, Puligundla et al., 2017). For instance, Zhou et al. (2016) found that air plasma produced through DBD microplasma array (4.5 kV; 25 W) offered the best efficiency in improving mung bean seed germination rate and seedling growth when compared to the control and O₂, N₂ and He microplasma arrays. However, contrary to the germination conditions used in this study (incubation of seeds in test-tubes at 25°C ± 1°C in the dark at 45% humidity level with water replacement after 48 h), Zhou et al. (2016) kept the seeds in Petri dishes, watered them daily and incubated them in light conditions. In another study, CAP produced using a DCSBD at 400 W with a plasma volume power density of 70 W/cm³ was employed to treat wheat seeds (Zahoranová et al., 2015). While these conditions are similar to those applied in this study, the wheat seeds were placed directly on the ceramic plate of the DCSBD device and fixed to a rotation device to homogeneously treat the seed surfaces (Zahoranová et al., 2015). Contrary to this, mung bean seeds assessed in this study were treated bi-directionally in a static position between two plasma plates kept at a distance of 1.5 cm each from the seeds. Furthermore, germination and growth of wheat seeds was assessed by sowing the seeds in pots containing soil substrate (a mixture of sand, peat and perlite). The wheat seeds exhibited a greater uptake of water than their untreated counterparts. In fact, following 20–50 s CAP-air treatment of seeds, significant accession of germination rate, dry weight and vigour of wheat seedlings was observed (Zahoranová et al., 2015). In comparing studies, germination conditions using light and soil are not realistically associated with sprout production practices. However, while taking into account that the closed germination set-up used in this study (test tubes) facilitated the microbiological assessments performed to determine the decontamination efficiency of combined plasma treated sprouts, the same system is not realistically comparable to common sprout production practices that enable the regular irrigation of sprouts. Furthermore, in comparing the technical plasma treatment parameters between studies, the distance of the seeds to the plasma source and associated treatment time during CAP treatment of seeds along with the frequency of irrigation with PAW may be key factors in regulating the level of exposure of seeds to reactive species that in turn determines the efficacy of plasma treatment on seed germination and growth.

Few studies have looked into the combined effect of direct CAP treatment of seeds and irrigation with PAW on seed germination and growth. In one study, Sivachandiran and Khacef (2017) elucidated the short term effects of combined plasma treatment on seed germination and stem growth of radish sprouts, and the long term effects of combined plasma treatment on tomato and sweet pepper plant growth. The study made use of a plate-to-plate DBD (operated with a high voltage pulsed power of 40 kV and frequency of 1 kHz) for the CAP treatment of seeds in air for 10 min (P 10) and 20 min (P 20) and a cylindrical DBD (40 kV; 1 kHz) for the cold atmospheric plasma activation of water in air for 15 (PAW 15) and 30 minutes (PAW 30). Seeds of the P 10 treatment irrigated with PAW 15 displayed better seedling growth when compared to the untreated seeds and to the P 10 treated seeds irrigated with tap water. Furthermore, non-treated seeds irrigated with PAW 30 performed better than non-treated seeds irrigated with tap water. Through this outcome, it was observed that aside from the stimulatory effect of plasma discharge on seed surface, sustained plasma treatment through irrigation of treated seeds with PAW can significantly enhance seedling growth. As may be applied in this study, achieving enhanced seed germination and seedling growth would require optimization of combined plasma treatment for each seed type, taking into account both gas phase characterisation and the physicochemical properties of the PAW (Sivachandiran and Khacef, 2017).

When assessing the inactivation of *E. coli* following exposure to PAW (air), PAW (N₂) and PAW (CO₂), it was found that although all three PAWs exhibited an acidic pH (3.34–3.91), only PAW (air) was found to inactivate *E. coli*. This suggests that an acidic environment is unlikely to be the sole sterilizing agent associated with PAW treatment, but that the type and

concentration of reactive species within the PAWs also play a significant role in determining its biological effect. In fact, peroxyxynitrite, a nitrogen containing reactive species, was previously found to play a crucial role in the antibacterial application of PAW (air) due to its cytotoxic effects (Ikawa et al., 2016, Zhou et al., 2018). Peroxyxynitrite is mostly formed by the reaction between H_2O_2 and NO_2 (Zhou et al., 2018). Theoretically, this reaction would not be possible in PAW (CO_2) for lack of nitrogen in the process gases, nor in PAW (N_2) for lack of reactive oxygen species such as H_2O_2 during plasma discharge. Further insight into the effect of the reactive species could be gained through independent inactivation assessments of *E. coli* following exposure to simulated aqueous concentrations of the reactive species identified and quantified in the PAW (e.g. H_2O_2 , NO_3^- , NO_2^- , ONOO^- , O_3 , and OH^-). Another study aimed to elucidate the bacterial cell damage caused to *Staphylococcus aureus* by oxidative stress from PAW generated in a single electrode alternating current cold plasma set-up (Zhang et al., 2013). Among the techniques used, atomic absorption spectroscopy detected an increased leakage of potassium ions from bacterial cytoplasm and transmission electron microscopy revealed morphological impairments to bacterial cell wall and membrane following exposure to PAW. The same paper also indicated the effect of short-lived species within PAW, noting that a bacterial suspension of *S. aureus* exposed to PAW directly after production and the same PAW stored in a 4°C refrigerator for 24 h after production required 10 min and 40 min, respectively, to achieve a 6 log reduction in the population of *S. aureus* (Zhang et al., 2013). The aforementioned techniques may also be employed to better substantiate and characterise the antimicrobial properties of PAW.

The lifetime of reactive species is an important parameter in understanding the long-term decontamination effects of PAW treatment on seeds. An initial screening on sprouts following CAP and PAW treatment of mung bean seeds indicated that CAP (air) treated seeds irrigated with PAW (air) demonstrated the greatest control over the natural microbiota (4.92 log CFU/g) of the sprouts after 96h in the incubation chambers (25°C) when compared to the control and other combined plasma treated groups (> 6 log CFU/g). This outcome potentially enhances the relevance of combined plasma treatment as a means of inhibiting bacterial proliferation during sprout production, which is stimulated by the warm temperatures, water activity and high nutritive content in the sprouts. The increased decontamination efficiency of the combined plasma treatment in air may correspond to the antimicrobial effect of PAW (air) determined in respect to *E. coli*, however further characterisation, primarily through identification of microorganisms is required. To the best of our knowledge, no previous studies have monitored the microbial population within sprouts following CAP and PAW treatment of seeds. However, recent studies have assessed the use of PAW as a means of decontaminating harvested mung bean sprouts with ancillary evaluation of the physicochemical characteristics of the sprouts following treatment. Schnabel et al. (2015) assessed the inactivation rates of *E. coli*, *Pseudomonas fluorescens*, *Pseudomonas marginalis* and *Pectobacterium carotovorum* in sprouts after 5 minutes immersion in air plasma processed water. The highest inactivation rates were observed for *E. coli* and *P. marginalis* while minimal effect on texture and appearance of the sprouts was recorded. More recently, Xiang et al. (2019) found that the total aerobic bacteria and yeasts within mung bean sprouts decreased by 2.32 and 2.84 log CFU/g, respectively following 30 min immersion of sprouts in air PAW. The authors also established that the washing treatment had no significant effect on antioxidant potential of mung bean sprouts and no changes in the phenolic and flavonoid contents nor sensory characteristics of the sprouts.

In the mung bean sprouts, kaempferol-3-rutinosid, quercetin-3-rutinosid, genistein, biochanin A and ononin derivative were tentatively identified. A review on the phytochemical profile of mung bean sprouts mentions other phenolic compounds and flavonoids present in the sprouts such as catechin, syringic acid, gallic acid, vitexin, robinin, kaempferol-7-O-rhamnoside and isoquercitrin (Ganesan and Xu, 2017) which were not found here. A variation of flavonoids and phenolic acids is common in *Vigna* species. In a previous study on pea sprouts exposed to CAP resulted in highest concentrations of quercetin and kaempferol glycosides, whereas the treatment of seeds and seedlings was not as efficient or even decreased quercetin and kaempferol glycosides (Bußler et al., 2015). Here all combined plasma treatments had no significant impact on the concentrations of secondary metabolites in the sprouts that gives a positive outcome on the application of cold plasma treatment in the agro-food industry.

Overall, this research demonstrated that air plasma activated water formed by 1 minute surface treatment with cold atmospheric plasma generated at 350 W (at a frequency of 15 kHz) in the DCSBD was able to reduce the population of *E. coli* DSM1116 by 7.43 log CFU/mL within 6 h of exposure. The combined seed treatment with direct air CAP (5 minutes, 350 W) and air PAW had no negative impact on mung bean seed germination and growth when compared to the control, nor was the concentration of secondary metabolites within the sprouts (kaempferol-3-rutinosid, quercetin-3-rutinosid, benzoic acid + malonic acid, ferulic acid + malonic acid and caffeic acid + malonic acid) reduced. The combined air CAP and air PAW treatment reduced the total microbial population in sprouts by 2.5 log CFU/g lower than the population of the control within 4 days, although further characterisation of the natural microbiota on the seeds prior to and after cold plasma treatment would allow for more accurate assessments on the effect of prolonged plasma treatment on the sprouts. The concentration of long-lived reactive nitrogen species, such as NO_3^- , NO_2^- are crucial in elucidating the complex mechanisms of action of PAW in disinfection and germination, along with the further characterisation of other reactive species such as hydrogen peroxide.

Materials And Method

Plasma Source

The operating conditions for the Diffuse Coplanar Surface Barrier Discharge (DCSBD) plasma system (CEPLANT, Brno, Czech Republic) used in this study were described in a previous study (Leslie, 2015) and are summarized below. This plasma system allowed for the operation of a homogeneous plasma produce in the presence of a process gas. For the purpose of this research three process gases were employed: air; N_2 ; and a mixture of 80% CO_2 with 20% O_2 . The DCSBD system consisted of 2 parallel opposing plates, the distance between which can be adjusted in the range of 0 to 30 mm. Strip-like electrodes (1.5 mm width, 0.5 mm thickness and 1 mm distance between electrodes) generate plasma on the surface of each plate. The electrodes do not come into contact with the plasma as they are separated by a 0.4 mm thick ceramic layer made of 96% alumina. A dielectric insulating oil circulation system was used for the electric insulation of the electrodes. The electrode arrangement of one of the plates is shown in Fig. 11.

With increasing power, a homogenous plasma layer is generated from a high number over an area of 200 mm x 80 mm on the electrode surface. The plasma has a thickness of 0.3 mm and operates through high frequency (15 kHz) sinusoids, with a voltage up to 20 kV (peak to peak) and power density up to 100 W/cm³. An external cooling system (Huber CC-410, Offenburg, Germany) is connected and set to 15°C to ensure the insulating oil temperature did not exceed 65°C.

Seed Treatments And Assessment Of Germination And Growth

Germination Set-up

A test tube set-up, adapted from Darmanin et al. (2020), was used for germination assessments. Test tubes filled with 9 g of glass beads were sterilized at 121°C for 15 minutes prior to use. One mung bean seed was inserted per tube and then irrigated with 3 mL (enough to partially immerse the seed) PAW or sterile distilled water (SDW) as the negative control. The tubes were placed in incubation chambers set at 25°C ± 1°C in the dark at 45% humidity level for 96 h. Following the first irrigation with SDW/PAW (0 h), the water in the tubes was replaced with fresh SDW/PAW at 48 h in order to re-enrich the irrigation system. The length of the sprout stem was measured 3–4 times a day using ImageJ software, from 48 h (i.e., time that sprouting was initiated) until 96 h to calculate growth rate and compare growth performance. Germination percentage at end point was also recorded. The aforementioned seed germination and growth assessment technique was applied for all plasma treatments described below.

CAP treatment of seeds

The first set of experiments was performed to assess mung bean seed germination and growth following seed treatment with CAP (refer to Fig. 12 (i)). The previously described DCSBD plasma device was used, where three CAP treatments were

assessed that varied by process gas: CAP (air) treatment infused the DCSBD chamber with 10 SLM air for 2 minutes, CAP (N₂) treatment infused the DCSBD chamber with 10 SLM N₂ for 2 minutes and CAP (CO₂) using a mixture of 80% CO₂ (2 SLM) and 20% O₂ (0.5 SLM) infused the DCSBD chamber for 5 minutes. For each treatment one sample set of 40 seeds (technical replicates) was treated bi-directionally in the DCSBD plasma chamber set at 350 W for 5 minutes at a distance of 1.5 cm both from the top and bottom plate. Single seeds were placed in 40 tubes, irrigated with SDW and incubated together with an untreated set which served as negative control.

PAW treatment of seeds

The second set of treatments assessed germination and growth of untreated seeds irrigated with 3 mL PAW (Fig. 12 (ii)). The same process gas parameters as described under CAP treatment of seeds were applied for the generation of three different PAWs: PAW (air), PAW (N₂) and PAW (CO₂). The DCSBD was set to 350 W for the surface CAP treatment of 50 mL SDW for 1 minute with a distance of 2 cm from the surface of the water to the top electrode. Following the treatments, pH and temperature of the PAWs was recorded. Hereafter, 40 tubes with single untreated mung bean seeds were irrigated with 3 mL of PAW and were incubated together with an untreated set that served as negative control.

Combined CAP and PAW treatment of seeds

Based upon assessment and evaluation of the individual effect of CAP and PAW treatments on mung bean growth rate and growth performance, one CAP treatment of seeds was combined with each of the three types for PAWs (Fig. 12 (iii)). The three combined plasma treatments assessed were: CAP (air) treated seeds irrigated with PAW (air), CAP (air) treated seeds irrigated with PAW (N₂) and CAP (air) treated seeds irrigated with PAW (CO₂). As all CAP treatments were performed in air, the annotations for the combined plasma treatments took on the following format: CAP + PAW (air), CAP + PAW (N₂) and CAP + PAW (CO₂). Each combined plasma treatment was assessed in triplicate on a set of 20 mung bean seeds. A triplicate set for the negative control was also performed simultaneously.

Determination Of The Antimicrobial Capacity Of PAW

The antimicrobial capacity of the PAWs was determined by assessing the inactivation of *Escherichia coli* DSM11116 over 6 h exposure to PAW. A two-step re-culturing of *E. coli* from a bead culture stored at -80°C was performed by transferring 1 bead to 5 mL Nutrient Broth (NB; ROTH, Karlsruhe, Germany). This suspension, termed as the pre-culture was vortexed and incubated at 37°C for approximately 24 h. The second sub-culture was incubated at 37°C for approximately 18 h until stationary phase was reached, whilst shaking at 165 rpm. A Multisizer (Beckman Coulter, California, U.S.) particle counter was used to obtain a starting concentration of approximately 10⁸ CFU/mL. Hereafter, 1 mL of the prepared culture was exposed to 9 mL of PAW while 9 mL sterile distilled water was used as a negative control. For each PAW sample, two technical replicate samples were analyzed after 0 (control only), 1, 2, 4 and 6 hours of PAW exposure. Analysis was performed by diluting the sample in Ringers solution inside a microtiter plate. Selected dilutions were plated on Plate Count Agar (PCA, ROTH, Karlsruhe, Germany). Plates were incubated at 37°C and bacterial counts were recorded after 24 h to calculate inactivation rate and compare log bacterial reductions between treatments. Apart from assessing two technical replicates from each sample, the procedure also assessed the antimicrobial capacity of three independent replicate samples of the control, PAW (air), PAW (N₂) and PAW (CO₂).

Determination of the microbial load of CAP and PAW treated sprouts

Microbial assessments were performed on sprouts grown from the combined plasma treatment of seeds (CAP and PAW) in order to determine the effect of plasma treatment on the natural microbiota of the sprouts. CAP treated seeds were assessed in order to determine the starting microbial population within both specimens. Untreated seeds were assessed as negative control. Germination was initiated following irrigation of seeds with respective treatment (SDW/PAW) in the test

tube set-up and incubated at $25^{\circ}\text{C} \pm 1^{\circ}\text{C}$ in the dark at 45% humidity level for 4 days. The microbial population in the sprouts was assessed on day 2, 3 and 4.

Approximately 5 g of seed and sprout samples were first homogenized in a 1/10 dilution in buffered peptone water (ROTH, Karlsruhe, Germany). From each homogenate, two technical replicate samples were analyzed. Analysis was performed by diluting the homogenate in casein peptone water (ROTH, Karlsruhe, Germany) inside a microtiter plate. Selected dilutions were plated on PCA and incubated at 30°C . Bacterial counts were recorded after 72 h in order to compare microbial load of the treated seeds and sprouts to the control. Apart from assessing two technical replicates from each homogenate, the procedure also assessed the decontamination efficiency of three independent replicate sample sets of control (seeds) and CAP treated seeds and control (sprouts) and combined plasma treated sprouts.

Quantification Of Secondary Metabolites

The method of extraction and quantification of secondary plant metabolites in the sprouts grown after 96 h of germination was adapted from Neugart et al. (2015). This involved dissolving 10 mg of freeze-dried sprouts in 600 μL 60% methanol and shaking for 40 min at 1400 rpm and 20°C in a thermal shaker. Centrifugation at 4500 rpm and 20°C for 10 min followed. The resulting supernatant was collected in a new reaction vessel. The pellet was dissolved again in 300 μL 60% MeOH and shaken at 1400 rpm and 20°C for 15 min. The sample was centrifuged at 4500 rpm and 20°C for 10 min. The supernatant was collected again. This step was performed twice. The collected supernatant was left to evaporate in the vacuum centrifuge until completely dry. The residue was dissolved in 200 μL 10% MeOH. The solution was then poured into Spin-X/Filter tubes with a 0.22 μm cellulose acetate membrane (Corning® Costar® Spin-X®, Sigma Aldrich Chemical Co., St. Louis, MI). These were then centrifuged at 3000 rpm and 20°C for 5 min. The remaining solution was then prepared for measurement with the high performance liquid chromatography mass spectrometry (HPLC-MS).

Flavonoid composition (including hydroxycinnamic acid derivatives and glycosides of flavonoids) was performed as previously described in Neugart 2017. The flavonoid glycosides were tentatively identified as deprotonated molecular ions and characteristic mass fragment ions, according to Schmidt et al. (2010) and Neugart et al. (2015) by HPLC-DAD-ESI-MSⁿ. For external calibration quercetin 3-glucoside and kaempferol 3-glucoside (Roth, Karlsruhe, Germany) were used curves in a semi-quantitative approach.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 8.0.1. The sprout stem length measurements recorded over time during the germination experiments were analyzed using the exponential growth equation. The exponential decay equation was used to analyze the log bacterial counts recorded over time during the PAW antimicrobial capacity experiments (Chap. 2.1.4.). Data sets including sprout stem growth rate, growth performance and germination percentage, log reduction of *E. coli* and log microbial population on sprouts were then tested for normality using the D' Agostino and Pearson test and the Shapiro Wilk test, performing data transformation when necessary. Statistical analysis using one-way ANOVA, two-way ANOVA and the Friedman test were performed to determine significance of treatment results compared to control results.

Declarations

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Author contributions statement

M.D. conducted the experiment, data analysis, writing. A.F. design experiments, interpretation of results. S.B. design experiments, interpretation of results, writing. J.D. design experiments, review & editing. S.N. Analysis of phenolic compounds, review & editing. M.S. Analysis of phenolic compounds, review & editing. R.B. conceptualisation, review & editing. R.G. conceptualisation, review & editing O.S. conceptualization, supervision, review & editing, funding acquisition. V.P.V. conceptualization, supervision, writing, review & editing, funding acquisition.

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