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FULL PAPER

PLASMA PROCESSES AND POLYMERS

Efficiency of plasma-processed air for biological decontamination of crop seeds on the premise of unimpaired seed germination

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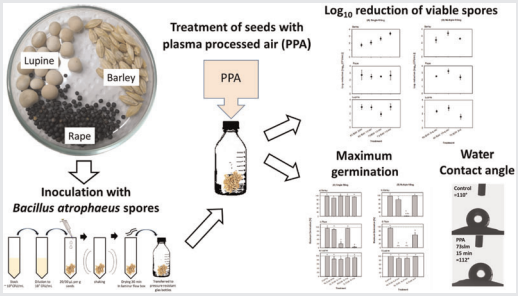
Abstract

In this study, the antimicrobial effect of plasma-processed air (PPA) generated by a microwave-induced nonthermal plasma was investigated for preharvest utilization using three crop species: Barley, rape, and lupine. *Bacillus atrophaeus* spores were chosen as a model, inoculated onto seeds, and subsequently treated with PPA at two different flow rates, different filling regimes, and gas exposure times. PPA treatment was efficient in reducing viable spores of *B. atrophaeus*, reaching sporicidal effects in all species at certain parameter combinations. Maximum germination of seeds was strongly reduced in barley and rape seeds at some parameter combination, whereas it had a modest effect on lupine seeds. Seed hydrophilicity was not altered. Overall, PPA investigated in this study proved suitable for preharvest applications.



KEYWORDS
decontamination, germination, NTP, plasma, plasma processed air, seeds

1 | INTRODUCTION

During the past years, increasing restrictions for the usage of chemical pesticides in seed and plant treatment in agriculture have been scheduled by European legislation. These restrictions are amongst others based on observed detrimental effects of those pesticides with respect to quantity and diversity of insects found in agricultural and rural environments.[1,2] A recent example is a ban of chemical seed dressing using the agent Thiram (TMTD), widely applied as a fungicide in rape and leguminous seed treatment to prevent soil-borne infections.[3] Therefore, preferably sustainable alternatives for seed treatment are urgently needed to secure crop productivity and yield.

One prospective and rather novel physical treatment is the application of nonthermal plasma (NTP). Plasma, in general, is considered as the fourth state of matter and contains charged particles (e.g., OH, H_2O^+, electrons,

etc.), reactive species (e.g., reactive oxygen species [ROS]: OH, O_2, $^1O_2^-$ and reactive nitrogen species [RNS], $NO^{\bullet}$, $ONOO^{\bullet}$, etc.), excited molecules (e.g., excited O_2, N_2, etc.), and UV photons (e.g., vacuum UV, UVC, UVB, etc.).[4,5] Furthermore, electrical fields and temperature are important components and influence the results of NTP treatments.[6,7] The mode of NTP treatment, in general, can be directly on the target or indirect via the generation of plasma-treated air (e.g., plasma-processed air [PPA]) or liquids (e.g., plasma-processed water), which are applied to the target[5,8–11] Direct NTP treatments have shown the potential for effective microbial decontamination and enhanced germination of different plant species with positive impacts on plant growth and development.[12–14] Antimicrobial efficiency of plasma has been tested on medical surfaces, food products and packaging, and on bulk goods and crop seeds.[15–19] Indirect treatment using processed air based on PLexc microwave plasma has proven efficient microbial decontamination at laboratory scale as well as at industrial scale on abiotic (e.g., glass, and plastic) and biological surfaces (e.g., fruit, vegetables, and meat).[20–27]

Damages caused to microorganisms most likely occur via ROS and RNS, targeting proteins and nucleic acids, as well as lesions in cellular membranes.[11,15,28,29] The main proposed mechanisms of bacterial inactivation include etching, electrophysical lysis, and electroporation. Biochemical mechanisms, which lead to cell death include modulation of biomembranes, such as deterioration of the cell membranes' integrity by oxidation of unsaturated fatty acids of the membrane lipids and oxidation of integrated membrane proteins of the double lipid layer, as well as impairment of proteins and whole-cell metabolism.[30–32]

Preharvest (period before crop harvest) application of NTP, unlike postharvest (stage of crop production immediately following harvest) has to fulfill the guideline to leave seeds with unimpaired viability to secure plant growth and yield. With regard to these prerequisites, in this study, the efficiency of PPA for decontamination generated by a microwave plasma was tested. At the same time, attention was paid to the viability of the treated crop seeds. Moreover, the impact of PPA treatment on seed surface hydrophobicity was studied. *Bacillus atrophaeus* spores were chosen as test organisms for artificial contamination of seeds surface. The spores of this Gram-positive bacterium is commonly used as a bioindicator for evaluating the efficiency of physical and/or chemical sterilization procedures in industry, biomedicine, and sanitation.[33] Its ubiquitous distribution in the natural environment and its harmlessness is of advantage because no specific safety measures are needed. Three crop plant species were chosen: *Hordeum*

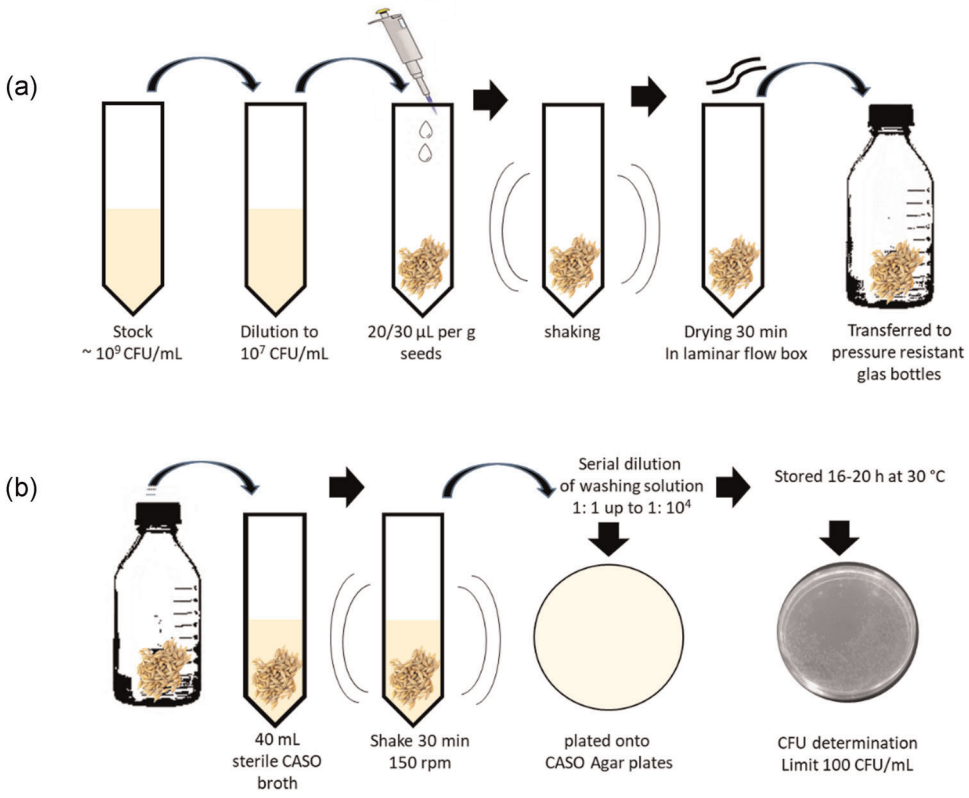


FIGURE 1 Schematic procedure of seed inoculation with *Bacillus atropheus* spore solution (a) and subsequent to plasma-processed air treatment, recovery of viable spores from the seed surface (b)

vulgare (food and feed grain,), *Brassica napus* (oil, protein-rich food, and feed, biofuel), and *Lupinus angustifolius* (protein-rich food and feed) with agricultural relevance and generally favorable processing and sales opportunities in Germany.

2 | MATERIALS AND METHODS

2.1 | Seed material

Seeds of *Hordeum vulgare* L. (barley) accession Kosmos were obtained from Ceravis AG, whereas seeds of *Brassica napus* L. (rape) accession Atora were provided by NPZ Innovation GmbH and seeds of *Lupinus angustifolius* L. (lupine) accession Boregine from Saatzucht Steinach GmbH.

2.2 | Artificial inoculation of seeds with *B. atrophaeus* spores and recovery from crops seeds

The sporulated form of *B. atrophaeus* (DSM 675) originated from the German collection of microorganisms and cell cultures (DSMZ); Braunschweig was used as a model in this study. A stock of spore suspension with a concentration of 10^9 CFU/ml was stored in sterile NaCl (0.85% w/v) at 7°C for further use. Inoculation of seeds was carried out using the diluted spore stock culture with a density of ~10^7 CFU/ml (Figure 1a). For each gram of seeds and a total of 10 g per replicate and tube, 20 µl of the diluted spore suspension was applied and vigorously mixed for barley and lupine seed and 30 µl for rape seeds by shaking for approximately 30 s to ensure an even distribution of spore solution. Seeds loading in total accounted for 6.2 ± 0.41 $\log_{10}$ CFU on 10 g of seeds for barley, $\log_{10}$ 5.4 ± 0.8 CFU per 10 g for rape, and $\log_{10}$ 5.15 ± 0.23 CFU per 10 g for rape seeds. Afterward, inoculated grains were dried for 30 min at room temperature in a laminar flow safety cabinet to allow the attachment of microorganisms on the seed surface. To evaluate the effect of PPA treatment, noninoculated grains were used and processed identically, without adding the spores to the solution. In preparation for treatment, seeds were transferred to pressure-resistant 1-liter glass bottles (Duran) with three replicates each.

After plasma treatment (see below), recovery of spores on the seed's surface was initiated by adding 40 ml of sterile CASO broth (Carl Roth) to the bottles (Figure 1b) and incubated for 30 min on a horizontal

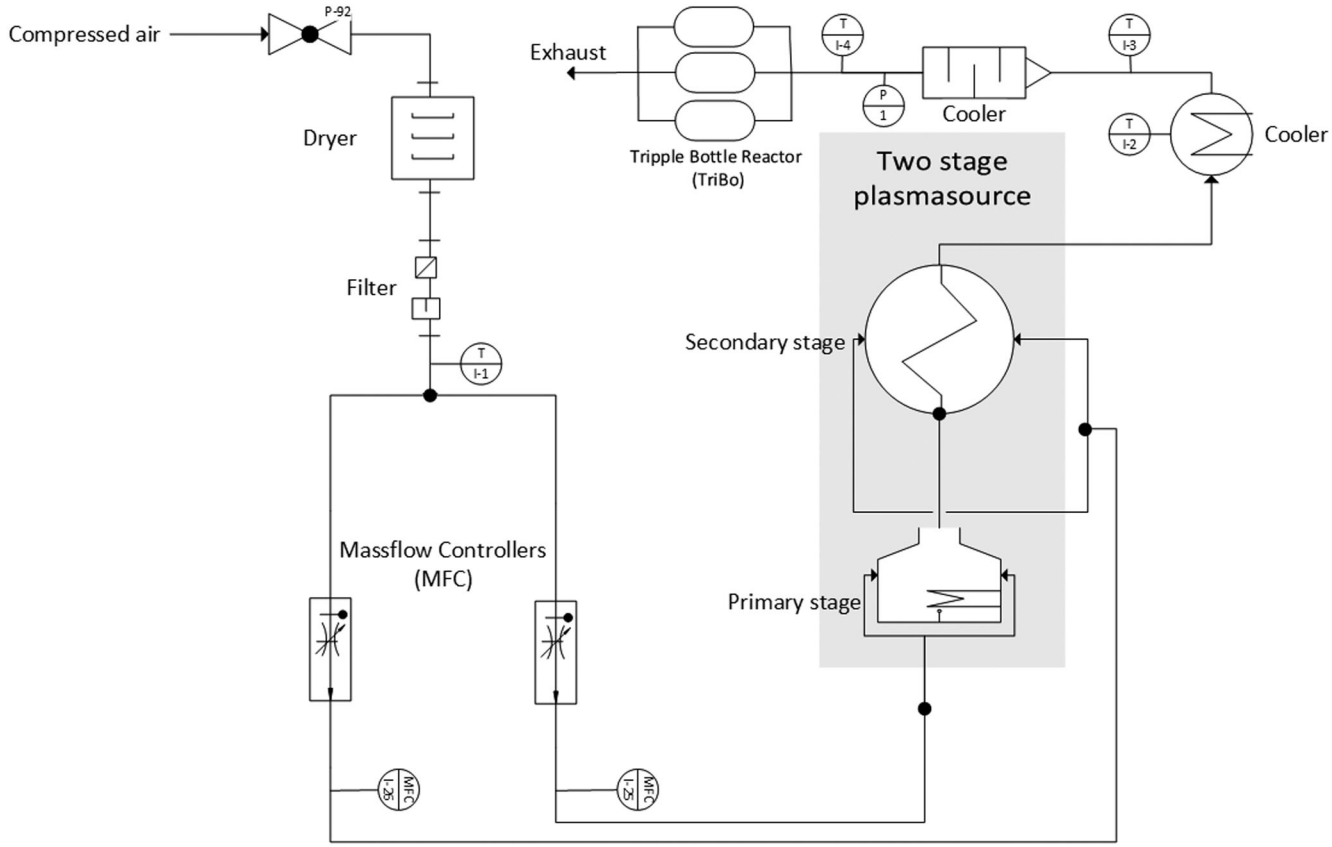


FIGURE 2 Scheme of the experimental setup for the generation of plasma-processed air, including the two-stage plasma source and the downstream triplicate bottle reactor for specimen exposure

shaker (150 rpm; IKA) at room temperature, followed by centrifugation and resuspension of the pellet. Resuspended pellets were serially diluted and aliquots (0.01 ml) of appropriate dilutions were plated on CASO agar plates (Carl Roth). Agar plates were incubated for 16–20 h at 30°C. Colony-forming units (CFU) were counted manually. Finally, the logarithmic reduction in CFU was calculated using the difference in the decimal logarithm of CFU/ml for PPA-treated seeds and the CFU/ml determined for untreated control samples.

2.3 | Generation of PPA and subsequent seed treatment

Figure 2 shows the process scheme and the setup generating and applying PPA. In the center of the schematic, highlighted in gray, the two-stage plasma source is shown. It is fed with dried compressed air via two mass flow controllers, which allow to adjust and control the gas flow through the two stages independently. After the two plasma stages, the process gas is cooled down and fed to the triplicate bottle reactor (TriBo) where the test specimens were treated according to the time schedule stored in the central control.

In more detail, PPA was generated by using a microwave-driven discharge[26] at a frequency of 2.45 GHz and the supplied power of 4 kW. Plasma was continuously operated during the pretreatment time, leading to a gas temperature of ~4000 K. During the exhaust gas transfer with a predefined gas flow from the plasma source into the glass incubation bottles, the gas further cooled down to room temperature (about 23°C). The applied gas volume for the treatment was 20 L for three 1-L glass incubation bottles, ensuring a complete gas exchange and filling with PPA. Once reaching the bottle, PPA was exposed to the seeds for different time intervals. To stop the posttreatment time, the incubation bottles were refilled twice with untreated compressed air.

The gas flow applied in this study was narrowed down to 63 and 73 slm, reverting back to previous results concerning biological decontamination in postharvest application.[21,27] For each of the three different plant species, three different plasma process parameters were investigated: Gas-filling mode, gas flow, and gas exposure time (Figure 3). The filling mode involved the single filling of glass bottles containing

Gas volume treated [L]	Gas flow [slm]	Filling method	Gas flow time [min]	Expoure time of processed gas [min]	Decontamination checked			Maximum germination checked		
					Barley	Rape	Lupine	Barley	Rape	Lupine
20	73	single		control				●	●	●
			-	1	●			●		
			-	2	●			●		
			-	3	●			●		
			-	5	●			●		
			-	7.5	●			●		
			-	10	●	●	●	●	●	●
			-	15	●	●	●	●	●	●
			-	17.5	●			●		
			-	20	●			●		
20	73	multiple	5	5	●	●	●	●	●	●
20	63	single	-	control				●	●	●
			-	3	●			●		
			-	5	●	●	●	●	●	●
			-	10	●	●	●	●	●	●
20	63	multiple	5	5	●	●	●	●	●	●
			10	5	●	●	●	●	●	●

FIGURE 3 Overview of experimental conditions used for the three different plant species

seeds and gas exposure for fixed time intervals in closed bottles, as well as multiple-filling, that is, gas flow of PPA for a certain time (gas flow and respective exposure time, see Figure 3) through the opened incubation bottles and subsequent gas exposure time in closed bottles. In the case of barley seeds, all combinations of plasma process parameters were checked for decontamination efficiency and maximum germination. For rape and lupine seeds, a smaller subset of plasma process parameters, was applied, as depicted in Figure 3. The experimental setup included two independent runs on different days with three to four replicates for each run, condition, and plant species.

2.4 | Seed germination tests

Maximum germination of PPA-treated and untreated seeds was surveyed in Petri-dishes mounted with layers of filter paper amended with 10 ml of tap water. Four replicates with each 50 seeds (barley and rape) or 30 seeds (lupine) were applied. Samples were incubated inside a climate chamber (Flohr Instruments; 12-h light/dark cycle; temperature 21°C during light phase and 18°C during dark phase). Germination was checked after 46/62 h for barley, 62 h for rape 62 h, and 52 h for lupine. A seed was considered as germinated once the radicle protruded ~1 mm in length. The estimated value was germination percentage: Max germination $\% = (n \times 100\%)/\mathrm{Nt}$, with n = number of seeds germinated and Nt = total number of seeds.

2.5 | Water contact angle (WCA) analysis

The surface hydrophobicity of all investigated plant species was examined by measuring WCA. To do so, the sessile drop method was applied after PPA treatment using a goniometer OCA 30 (DataPhysics Instruments) on selected process parameters (63-slm, single-filling, 5-min gas exposure time and 73-slm, single-filling, 15-min gas exposure time). The seeds were glued on top of a flat surface with the crease facing down. Three drops of 2.0 µl of deionized water were placed on top of each seed on the convex side, and for evaluating the software SCA 20 (DataPhysics Instruments) was used. For each treatment parameter, a set of 10 seeds was analyzed.

2.6 | Statistical analysis

All statistical analyses were done using SigmaPlot 13 (SigmaPlot). To compare mean values of maximum germination and water contact angle of PPA-treated and untreated control seeds, Student's t test was applied. For decontamination efficiency of PPA, a regression analysis was performed for $\log_{10}$ reduction in viable spores on the extended set up of barley. In addition, to test the impact of PPA process parameters and their interacting effects on $\log_{10}$ reduction and maximum germination, a one-way analysis of covariance (ANCOVA) on the treatments with "filling mode" or "gas flow" as a factor and "gas flow" or "gas exposure time" and "filling mode" as covariates was performed.

3 | RESULTS

3.1 | Efficiency of PPA treatment to inactivate inoculated bacteria spores

The survey of decontamination efficiency of PPA on seeds of three plant species yielded in a mean reduction of viable *B. atrophaeus* spores for single filling and 63-slm gas flow of 1.95 ± 0.36 $\log_{10}$ (CFU/ml) in barley, 2.59 ± 0.74 $\log_{10}$ (CFU/ml) in rape, and 2.91 ± 0.48 $\log_{10}$ (CFU/ml) in lupine seeds, respectively (Figures 4a,4c, and 4e). Single filling and 73-slm gas flow resulted in a mean reduction of 3.00 ± 0.33 $\log_{10}$ (CFU/ml) in barley, 2.56 ± 0.61 $\log_{10}$ (CFU/ml) in rape, and 2.44 ± 0.52 $\log_{10}$ (CFU/ml) in lupine seeds, respectively (Figures 4a,4c, and 4e). Minimum reduction at single filling was 0.18 $\log_{10}$ (CFU/ml) in barley, 1.98 $\log_{10}$ (CFU/ml) in rape, and 1.21 $\log_{10}$ (CFU/ml) in lupine seeds.

For the multiple-filling regime, mean reduction of viable spores for both gas flow regimes accounted for 2.81 ± 0.53 $\log_{10}$ (CFU/ml) in barley, 2.67 ± 0.49 $\log_{10}$ (CFU/ml) in rape, and 2.27 ± 0.63 $\log_{10}$ (CFU/ml) in lupine seeds, respectively (Figures 4b,4d, and 4f). Minimum reduction was 2.6 $\log_{10}$ (CFU/ml) in barley, 2.03 $\log_{10}$ (CFU/ml) in rape, and 1.6 $\log_{10}$ (CFU/ml) in lupine seeds.

Statistical analysis combining ANOVA and regression analysis, that is, ANCOVA indicated for barley seeds that the factor "filling regime" and the covariate gas flow had no significant impact on decontamination efficiency, whereas the covariate gas exposure time turned out to be a significant covariate ($p = .02$, Table 1). The interactions of factors and covariates did not significantly affect decontamination efficiency. For rape and lupine seeds, no effect of the factor filling regime nor the covariates gas flow and gas exposure time or their interactions was observed (Table 1).

The more detailed analysis of decontamination effect of PPA, including a wider range of gas exposure times for the single-filling mode for barley seeds displayed an exponential increase in $\log_{10}$ reduction with increasing gas

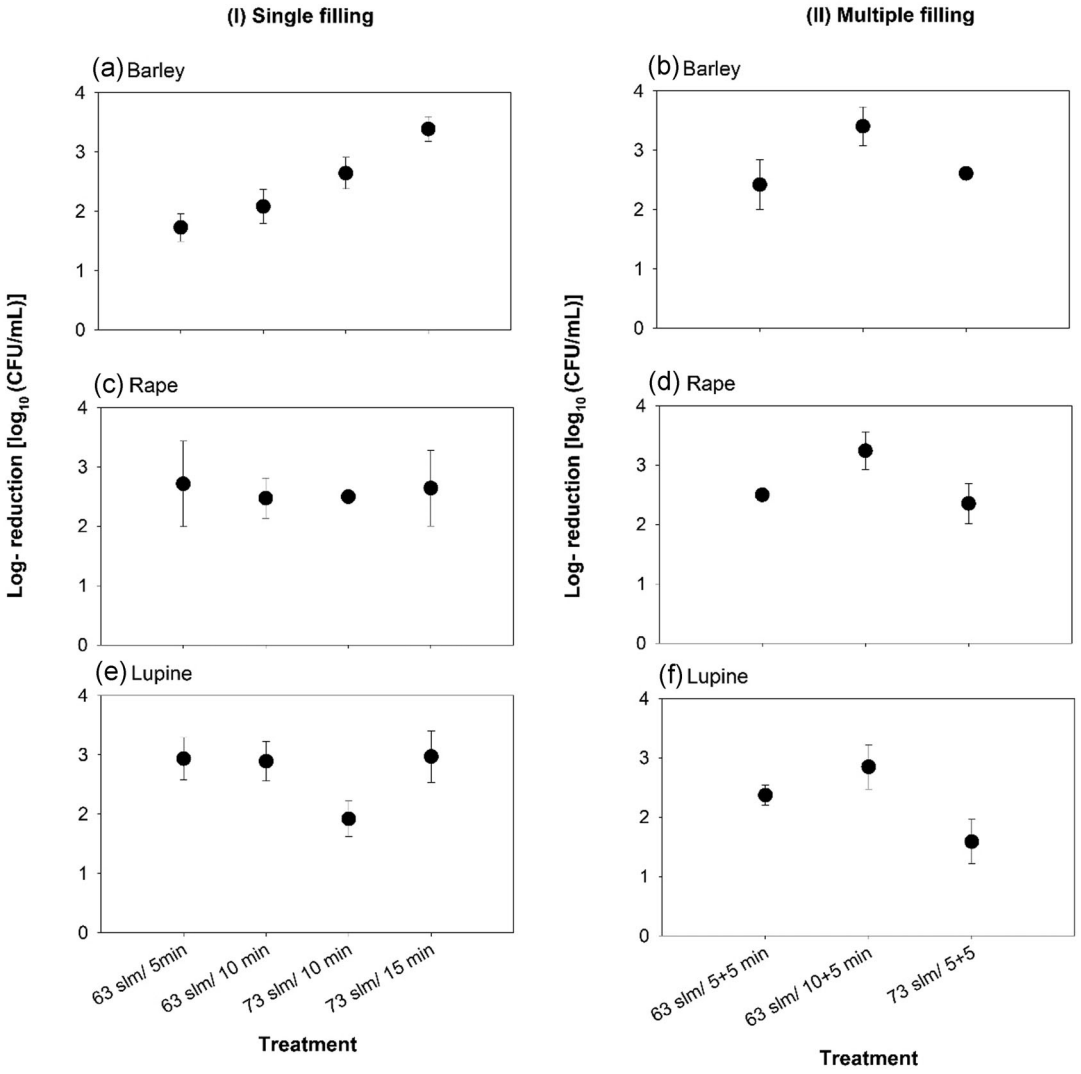


FIGURE 4 Log reduction in viable *Bacillus atrophaeus* spores across all plant species for (I) single filling of incubation bottle with plasma-processed gas and (II) multiple filling of incubation bottle with plasma-processed gas

exposure time for gas flow of 73 slm (Figure 5a). For 63-slm gas flow, fewer data points of gas exposure times were screened. Those parameters investigated yielded a linear rise in $\log_{10}$ reduction up to the gas exposure time of 10 min (Figure 5b).

For the extended data set, ANCOVA analysis revealed that the factor gas flow significantly affects the values of the log $_{10}$ reduction ($p = .006$, Table 2). Moreover, there was a significant interacting effect between the factor "gas flow" and the covariate "gas exposure time" ($p = .022$, Table 2).

3.2 | Impact of PPA treatment on seed viability

PPA treatment showed negative impacts on maximum germination for some process parameters (Figure 6), whereby the degree of impairment was depended on the plant species tested.

In barley seeds, two-parameter combinations led to a drastic drop in maximum germination compared to the untreated control by 97% (5 + 5 min) and 18% (5 + 10 min) at multiple-filling only and 63 slm gas flow (Figure 6a,b). At 73 slm and single-filling, a drop in maximum germination relative to control was detectable by 4%.

The most dramatic drop in germination occurred for rape seeds with a complete loss of germination (0%) in two-parameter combinations for multiple filing and 63-lm gas flow, as well as a drop by 91% at single-filling, 73-slm and 15-min gas exposure time (Figure 6c,d). The reduction in germination was more pronounced at 63 slm and for longer gas exposure times.

In lupine seeds, the highest reduction in maximum germination 8% to 15% occurred mostly at 63-slm gas flow (Figure 6e,f).

TABLE 1 Results of one-way ANCOVA analysis of decontamination for the three tested species using "filling mode" as a factor and "gas flow" and "gas exposure time" as covariates (compare Figure 4)

Plant species	Variables	*df*	*F*	*p* Value
Barley	Filling mode	1	0.194	.670
	Gas flow	1	0.003	.958
	Gas exposure time	1	8.016	**.020**
	Filling mode × gas flow	1	0.402	.542
	Filling mode × exposure time	1	0.200	.665
Rape	Filling mode	1	0.036	.88
	Gas flow	1	0.012	.930
	Gas exposure time	1	5.405	.259
	Filling mode × gas flow	1	0.057	.851
	Filling mode × exposure time	1	6.84	.232
Lupine	Filling mode	1	0.038	.877
	Gas flow		2.569	.355
	Gas exposure time	1	1.067	.490
	Filling mode × gas flow	1	0.028	.893
	Filling mode × exposure time	1	7×10^{-4}	.982

Note: Bold numbers indicate a significant effect and interaction on decontamination to level $p \leq .05$.

Abbreviations: ANCOVA, analysis of covariance; *df*, degree of freedom.

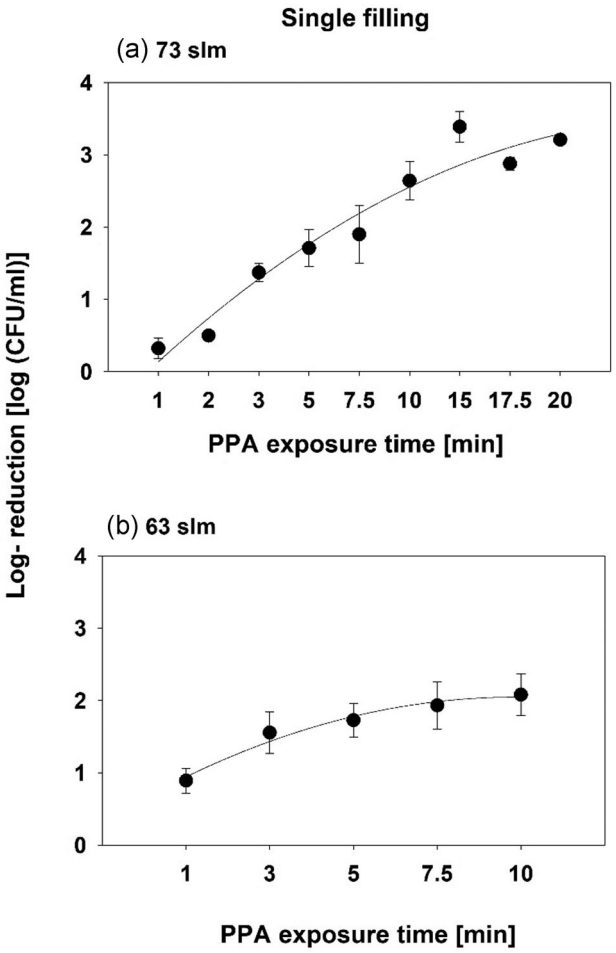


FIGURE 5 Extended data set on log reduction of viable *Bacillus atrophaeus* spores on barley seeds for a single-filling regime of incubation bottle with plasma processed gas at a flow rate of (a) 73 slm and (b) 63 slm. The solid lines represent fit of data with (a) exponential regression $R^2 = 0.944$, $F = 145.3$, $p \leq .0001$, $n = 54$) and (b) linear regression $R^2 = 0.995$, $p < .029$, $n = 18$)

TABLE 2 Results of one-way ANCOVA analysis of decontamination for the extended data set using barley (Figure 5)

Plant species	Variables	*df*	*F*	*p* Value
Barley	Gas flow	1	13.18	**.006**
	Gas exposure time	1	3.77	.088
	Gas flow × exposure time	1	8.00	**.02**

Note: "Gas flow" was used as a factor and "gas exposure time" as a covariate. Bold numbers indicate a significant effect and interaction on decontamination to level $p \leq .05$.

Abbreviations: ANCOVA, analysis of covariance; *df*, degree of freedom.

For barley, ANCOVA revealed that maximum germination was statistically affected by gas flow and gas exposure time as covariates ($p \leq .001$ in both cases), as well as by the interaction with factor "filling regime" ($p = .003$ and $p \leq .001$, respectively, Table 3). Filling the regime alone did not have a statistically significant effect. No impairment of germination was visible in the following process parameter combination: Single-filling, 63- and 73-slm gas flow, 10-min gas exposure time, as well as for multiple-filling, 73-slm gas flow, and 5-min gas exposure time.

For rape seed, no statistically significant effect was detected for the factor "filling mode," and the covariates "gas flow" and "exposure time" nor for their interaction. On the contrary, a complete loss in germination was observed at both filling regimes and affected germination in the same manner, with each at least one parameter combination for both filling regimes and gas flows leading to the loss of germination. No impairment of germination was only visible in one process parameter combination: Single-filling, 73-slm gas flow, and 10-min gas exposure time.

Negative effects in lupine were not restricted to a certain filling mode or gas flow, leading to no statistically significant impact of factor and covariates (Table 3). No impairment of germination was visible in

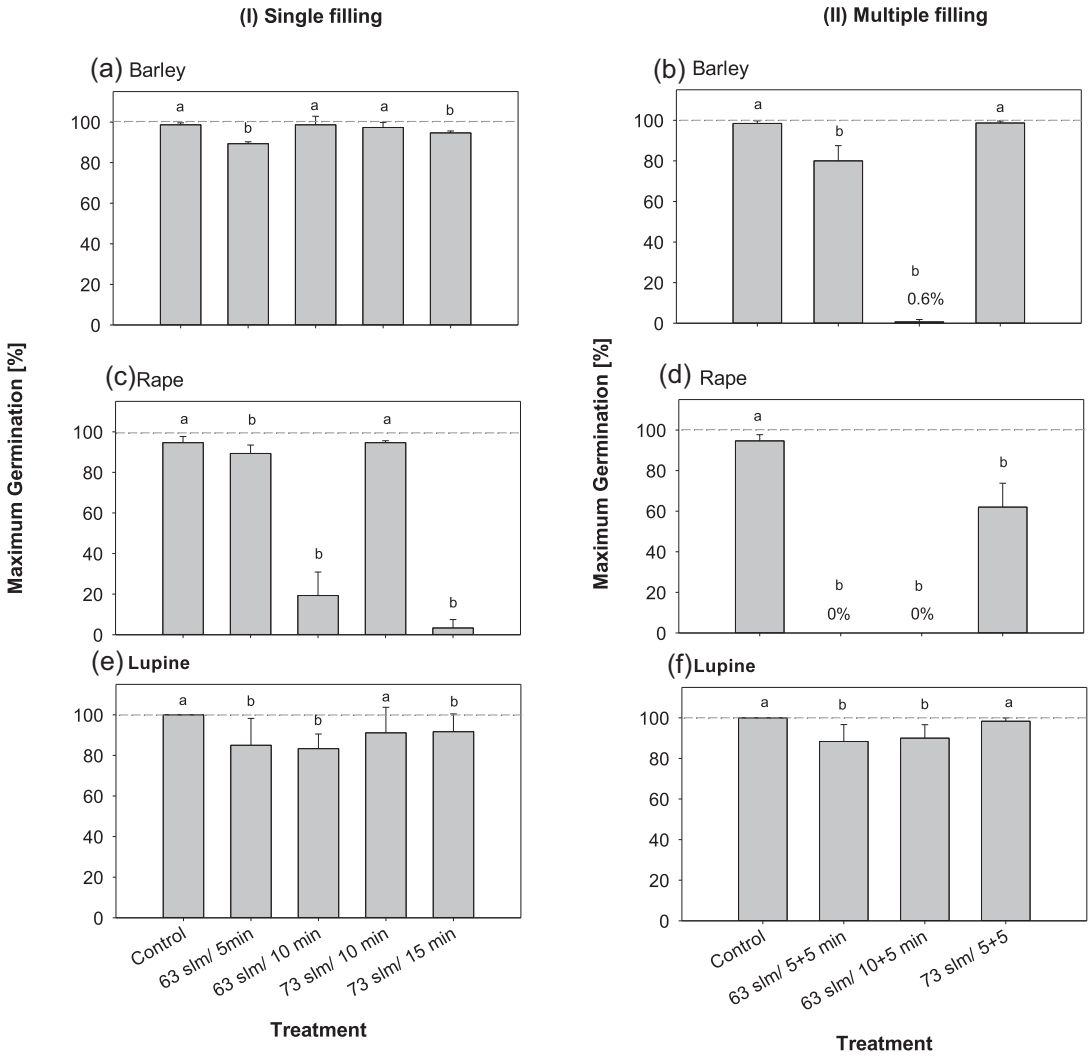


FIGURE 6 Maximum germination for the three plant species tested using single filling (I) and multiple filling (II) of incubation bottles and different gas exposure times and gas flow. Different letters from the control indicate significant deviation according to Student's *t* test with $p \leq .05$

the following process parameter combination: No impairment of germination was visible in the following process parameter combination: Single-filling, 73-slm gas flow, 10-min gas exposure time, and multiple-filling, 73-slm gas flow, and 5-min gas exposure time.

A higher resolution of different gas exposure times for single filling and 73 slm, as well as 63-slm gas flow, was investigated for barley (Figure 7). The results indicate only slight effects on maximum germination with no significant negative effect for short gas exposure times at 73-slm gas flow up to 10 min (Figure 7a). For gas flow of 63 slm, a decrease in maximum germination at 3-min (4%) and 5-min (9%) gas exposure time was visible, but no impairment was detectable at 10 min (Figure 7b), respectively, compared to untreated control seeds. Analysis of covariance indicated, gas flow as a factor significantly affected maximum germination, also in interaction with gas exposure time as a covariate ($p = .006$ and .022, Table 4).

3.3 | Impact of PPA treatment on seed surface hydrophobicity

Contact angle measurements were applied to determine whether plasma treatment affects the seed's surface characteristics for the three different plant species used. Apparent water contact angles (WCA) were not significantly different comparing untreated control seeds and the two types of PPA treatments. Moreover, WCA did not deviate in-between the species examined (Table 5).

TABLE 3 Results of one-way ANCOVA of maximum germination for the three tested species using "filling mode" as a factor and "gas flow" and "gas exposure time" as covariates (compare Figure 6)

Plant species	Variables	*df*	*F*	*p* Value
Barley	Filling mode	1	1.28	.286
	Gas flow	1	31.76	**<.001**
	Gas exposure time	1	503.96	**<.001**
	Filling mode × gas flow	1	15.65	**.003**
	Filling mode × exposure time	1	491.27	**<.001**
Rape	Filling mode	1	0.409	.638
	Gas flow	1	41.441	.098
	Gas exposure time	1	19.064	.143
	Filling mode × gs flow	1	0.391	.644
	Filling mode × exposure time	1	19.064	.143
Lupine	Filling mode	1	0.68	.559
	Gas flow	1	64.00	.079
	Gas exposure time	1	0.333	.667
	Filling mode × gas flow	1	1.000	.500
	Filling mode × exposure time	1	1.333	.454

Note: Bold numbers indicate a significant effect and interaction on decontamination to level $p \leq .05$.

Abbreviations: ANCOVA, analysis of covariance; *df*, degree of freedom.

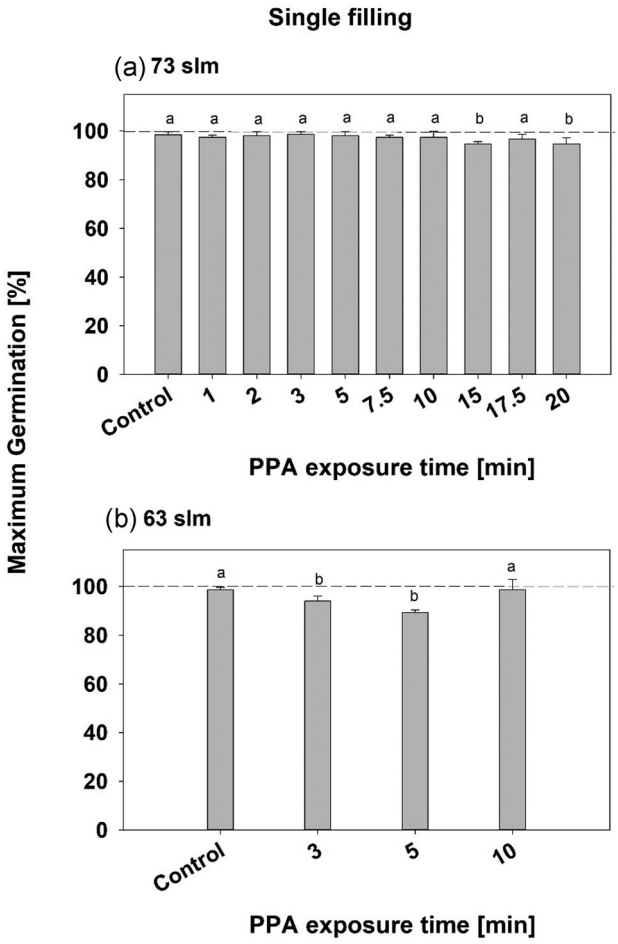


FIGURE 7 Extended data set on maximum germination for barley, using a single filling of incubation bottles with a gas flow of 73 slm (a) and 63 slm (b) and different gas exposure times. PPA, plasm-processed air. Different letters from the control indicate significant deviation according to Student's *t* test with $p \leq .05$

4 | DISCUSSION

4.1 | Decontamination of seed surface by PPA

The decontamination efficiency of PPA against artificially inoculated bacterial spores onto the seed surface of barley, rape, and lupine was evaluated in this study. The maximum reduction of viable spores for barley, rape, and lupine seeds accounted for 3.38 ± 0.21 $\log_{10}$ CFU/ml, 3.24 ± 0.24 log $\log_{10}$ CFU/ml, and 2.97 ± 0.23 $\log_{10}$ CFU/ml. These results confirmed that the previous data were obtained for different microorganisms, including spore-forming bacteria, on various plant seeds.[24,34–38]

The sporicidal effect of NTP, in general, is presumably based on physical mechanisms, including etching, electrophysical lysis, and electroporation, as well as biochemical mechanisms, including modulation of whole-cell metabolism (e.g., Liao et al.[30] and references therein). For PPA investigated in this study, chemical modifications and biochemical mechanisms are the main effects because UV-light and etching are unlikely to interact with the target during indirect treatment. This has been already shown by Ikawa et al.,[39] stating that for inactivating *Escherichia coli* and *Leuconostoc citreum* using indirect plasma treatment, there is most likely a critical pH ~4.7, below which efficient inactivation occurs. Moreover, the authors demonstrate, that not UV light, nor temperature control the bactericidal effects, but

TABLE 4 Results of one-way ANCOVA analysis of maximum germination for the extended data-set using barley

Plant species	Variables	*df*	*F*	*p* Value
Barley	Gas flow	1	13.81	**.006**
	Gas exposure time	1	13.77	.088
	Gas flow × exposure time	1	8.00	**.022**

Note: "Gas flow" was used as a factor and "gas exposure time" as a covariate (compare Figure 7). Bold numbers indicate a significant effect and interaction on decontamination to level $p \leq .05$.

Abbreviations: ANCOVA, analysis of covariance; *df*, degree of freedom.

TABLE 5 Effect of two different plasma-processed air treatments exemplarily on the water contact angle (WCA) of the seed surface for the three plant species studied

Plant species	Treatment	WCA (°)
Barley	Control	110.1 ± 9.5
	63-slm, single-filling, 5-min gas exposure time	113.5 ± 5.5
	73-slm, single-filling, 15-min gas exposure time	112.2 ± 7.0
Rape	Control	106.4 ± 1.6
	63-slm, single-filling, 5-min gas exposure time	110.5 ± 5.6
	73-slm, single-filling, 15-min gas exposure time	108.5 ± 5.5
Lupine	Control	118.8 ± 10.8
	63-slm, single-filling, 5-min gas exposure time	118.2 ± 6.2
	73-slm, single-filling, 15-min gas exposure time	120.9 ± 6.2

Note: No significant difference to control at the level of $p \leq .05$ according to Student's t test was detected.

highly reactive species presumingly generated in the solution via plasma–liquid interaction.[39]

Studying the inactivation effect of atmospheric pressure dielectric barrier discharge (DBD) on *Geobacillus stearothermophilus* spores, Butscher et al.[40] excluded mechanical and electrical stress factors and proposed chemical sputtering, which induces erosion of the seed's surface due to chemical sputtering as the predominant inactivation mechanism, along with potential effects of UV photons. Using Raman spectroscopy and phase-contrast microscopy, Wang et al.[32] concluded that bacterial sprees lose their ability to germinate and, therefore, reproduction caused by damaging the inner spore membrane and key germination proteins. Moreover, the authors identified charged particles and ROS as a key component for inactivation, whereas UV photons contributed to a minimal degree, deduced from the fact that UV-A had been minimized by using plastic bags. The experimental setup in our study most likely generated preferentially RNS and only a smaller amount of ROS.[41]

In general, several mechanisms of NTP microbial spore inactivation have been proposed in the past, which include: Damage of essential spore proteins involved in spore germination, lipid peroxidation resulting in impairment of the inner membrane, including its permeability or destruction of spore DNA.[30,32,42]

PPA, as applied in this study, is composed of high RNS, including nitride oxides and its possible reaction compounds nitrogen dioxide, nitrous acid, and nitric acid. Those components form an acid environment known for favoring the inactivation of spores.[41]

In our study, the efficiency of decontamination seemed to be dependent on the plant species. For barley, a strong dependency of gas flow and the exposure time was detectable, which might result from their uneven seed geometry and rough surface caused by the husk hull. Bacterial spores might get stuck in substructures on the seed surface and escape from efficient inactivation. For rape and lupine, no strong dependency of microbial decontamination efficiency on plasma process parameters was observed, hinting at efficient inactivation of even and round structure with little surface roughness. These effects have been observed and published before, including more efficient decontamination of PET film compared to Tychem F (laminate containing Tyvek® with microstructures),[25] as well as flat and granular polypropylene substrates in contrast to wheat grains.[43] Moreover, plasma inactivation levels of the fungal load were more pronounced for smoother corn seeds compared to barley seeds.[44] Associated with seed topography and structure, the penetration ability of plasma/PPA into gaps has to be mentioned. Sporicidal effects of plasma seem to be affected by the pore size and depth/width ratio of the object treated.[19]

It has to be noted that our study was performed on artificially inoculated seeds. A diverse native microflora, including naturally occurring pathogens (e.g., *Tilletia caries* causing common bunt in cereals and *Colletotrichum lupini* causing anthracnose in legumes) may pose a greater resistance to surface decontamination approaches, which warrants investigation in the future.

4.2 | Viability of seeds after PPA treatment

Seed viability was equally important as decontamination efficiency by PPA treatment in this study. Irrespective of sporicidal effects observed for certain PPA parameters, an impairment of seed viability would entail disregarding this plasma process parameter for future application. In our study, the seed viability was only ensured in some cases of a plasma process parameter, especially with rape seeds being most sensitive to PPA treatment. Maximum germination for barley was strongly negatively affected at two plasma parameter combinations (multiple-filling, 63-slm, 10 + 5-min) and mildly negatively affected by three other treatment conditions. Compared to

maximum germination in the untreated control seeds for rape seeds, a dependency in the reduction of germination on gas flow and the gas exposure time was identified. Thus, multiple-filling regimes and longer PPA exposure times affected rape seed germination most significantly. The impediment of rape seed germination compared to untreated control seeds occurred more often at the slower gas flow rate (63 slm). Air with slower flow rates has a longer exposure time during passing the microwave plasma source devise and therefore can, in principle, absorb more energy, resulting in possibly higher concentrations of RONS in the effluent air. However, Schnabel et al.[24] found no negative effects of indirect PPA treatment on rape seed germination using a microwave setup with a gas flow of 20 slm and a post-treatment gas exposure time of 15 min.

Lupine seed germination was only mildly negatively affected with a decrease by not more than 20% of maximum germination in five plasma process parameter combinations compared to the untreated control.

These effects warrant further investigation to elucidate the underlying mechanisms of loss in seed viability by different PPA treatment parameters. Possible reasons might include a strong reduction in pH by the formation of chemicals that harm seed germination or due to the interaction of plasma components and reaction products with seed surface molecules. PPA produced by a similar setup used in this study reduced the pH below 3, when applied to water.[45,46]

Thus, the observed negative effects might also depend on the moisture content of seeds because PPA–liquid interactions result in various physical and chemical processes.[47] Moreover, Patil et al.[48] suggested that high humidity contributes to the formation of reactive species other than ozone, which might interact with the target. In our study, seed moisture content was constant and within tolerable ranges of seed storage guidelines for the seeds tested (≤18%)[49] and most likely did not provoke a significant negative effect in combination with PPA treatment on germination.

Second, distinctions in the reduction of germination after PPA treatment might result from differences in seed coat structure and seed size, which determined the surface area to volume ratio during exposure. In general, smaller seeds display less volume in relation to their surface area. Rape seeds represented the smallest seeds tested with on average 0.3 cm in diameter for the accession tested and were also the ones displaying the most negative impact of PPA exposure. Also, the reactivity of PPA might decay more slowly during the exposure time when incubating smaller seeds due to a less pronounced reaction between reactive components in the gas and the seed surface.

Significant larger seeds were represented by barley, which displayed an average length of 0.9 and 0.3 cm in width, whereas more round-shaped lupine accounted for a diameter of 0.7 cm on average for the accession tested.

In addition to seed size, the seed coat structure differs between the seeds tested, which might also provoke different degrees of vulnerability leading to a loss in germination after PPA exposure. The least complex structure is represented by rape seeds, which display a testa with thickness of 50 μm on average comprising the substructures of epidermis, palisade, and aleuronic layer.[50] More complex seed coats are found in barley and lupine seeds. At least four outer tissue layers are present in lupine seeds with a thickness varying from around 170 to 335 μm, depending on genotypes.[51,52] Barley used in our study is covered to different extents by the hull (husk) and possesses a pericarp, which is fused to the outside of the true seed coat (testa).[53,54] According to micrographs, these multilayered structures can be estimated with approx. 70- to 100-μm thickness, surrounding the endosperm and germ.[55–57] Overall, lupine as well as barley seeds seemed to be more protected against detrimental effects of PPA treatment.

Future investigations should include seed germination and plant growth in soil substrates to apply a more natural environment where multiple factors will come into play (e.g., buffering substrate, ions of substrate polymers, water, and temperature). Negative effects on seed germination, which were detected for some of the PPA parameters, tested might be reversed under varying factors mentioned above.

DBD of wheat seeds displayed stable maximum germination up to an exposure time of 20 min.[34] Inhibition in seed germination was also noted in the past and was associated to seed coat hardness and thickness of the endosperm.[58,59]

In recent years, numerous publications proposed enhancement of seed germination and seedling growth rates after direct NTP treatment, often associated with an improved water uptake of seeds,[60,61] structural and morphological changes on seed surfaces,[59,61,62] or due to an interplay with the RONS generated by the plasma.[34,58,63–66] Direct plasma treatment is changing the WCA and seed hydrophilicity to a great degree, originate from the incorporation of oxidized functional groups on the seed surface[61] and often leading to accelerated germination.[34,67–69] Our study, on the contrary, indicated that surface hydrophilicity of barley, rape, and lupine seeds were not changed by PPA treatment as concluded from the unchanged WCAs for each plant species. This different impact of direct and indirect plasma treatment on WCA has been noted before.[34]

TABLE 6 Summary of plasma process parameter and their correlation with decontamination efficiency and maximum germination according to analysis of covariance

Plant species	Plasma process parameter	Decontamination efficiency	Maximum germination	Reduction in maximum germination ≥ 80%	Optimal plasma process parameter
Barley	Filling mode	✗	✗	Multiple	Single
	Gas flow (slm)	✓	✓	63	73
	Gas exposure time (min)	✓	✓	10 + 5	10
Rape	Filling mode	✗	✗	Single, multiple	Single
	Gas flow (slm)	✗	✗	63, 73	73
	Gas exposure time (min)	✗	✗	10, 15, 5 + 5, 10 + 5	10
Lupine	Filling mode	✗	✗	-	Single
	Gas flow (slm)	✗	✗	-	73
	Gas exposure time (min)	✗	✗	-	10

Note: In addition, the plasma process parameter is shown, which induced a decrease in maximum germination by 80% or higher, as well as the optimal plasma processes combination. ✓, statistical effects detected; ✗, no significant impact.

5 | CONCLUSION

Indirect plasma treatment using the Plexc setup in this study proved suitable to preharvest applications for barley, rape, and lupine. The process parameter was evaluated according to the sporicidal effect (≥3 $\log_{10}$ units reduction) combined with unimpaired maximum germination (Table 6). For rape and lupine, the decontamination efficiency appeared relatively stable over all process parameters with no correlation to the process parameter. A sporicidal effect and reduction of spores by 99.9% and beyond were achieved. For barley seeds, increasing gas exposure time resulted in rising decontamination efficiency, also reaching sporicidal values. In contrast to this, germination and seed viability seemed to be more sensitive to certain PPA parameter combinations. Especially, rape seeds displayed up to complete loss of germination in four PPA parameter combinations that displayed a trend which seemed to be dependent on the gas flowrate compared to the germination of untreated control seed. In lupine seeds, reduction in maximum germination was less pronounced and mostly occurred at a lower gas flow rate, whereas in barley seeds the reduction significantly correlated with exposure time and gas flow. In all three plant species, the optimal process parameter combining sporicidal effect and unimpaired germination was identified as single-filling mode, 73-slm gas flow, and 10-min gas exposure time. WCA as a proxy for seed surface hydrophilicity was not affected by PPA treatment. In conclusion, to prevent losses in seed viability, the plasma process parameter has to be evaluated for each species individually to exclude detrimental combinations. The technical implementation of Plexc, including scale-up, is likely feasible by increasing the volume of applied gas and the usage of a corresponding gas-tight container for treatment of large amounts of seeds.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding authors upon reasonable request.

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