**Taheri, Saeedeh, Brodie, Graham Ian, Gupta, Dorin, and Jacob, Mohan V. (2020)** ***Afterglow of atmospheric non-thermal plasma for disinfection of lentil seeds from Botrytis grey mould.*** **Innovative Food Science & Emerging Technologies, 66 .**



https://doi.org/10.1016/j.ifset.2020.102488

# Afterglow of atmospheric non-thermal plasma for disinfection of lentil seeds from Botrytis Grey Mould

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## Abstract

Seed-borne fungal diseases of grains are a serious threat to a crops' yield due to lack of either resistant crop varieties or dependence on fungicides. Therefore, there is a growing demand to develop sustainable technologies for crop protection. In the present study, an atmospheric microwave plasma was employed to eradicate *Botrytis* from lentil seeds. Argon, air and a combination of them were applied to create the plasma. There was a 41% reduction in the percentage of inoculated seeds (with an initial contaminated seed percentage of 95.8%) after 100 s treatment of 2.5 g of lentil seeds with the afterglow of air plasma followed by 24-h holding time. A 32.3% reduction occurred when a 30% air/70% Argon was applied for 10 s and 60 min of holding time. The holding time of 24 h increased catalase activity from 0.8 to 1.1 mM $H_2O_2$ mg$^{-1}$ min$^{-1}$ that was an indicator of early plant immune system fortification. This also changed seeds' colour toward redness and yellowness. Conclusively, the afterglow of microwave plasma could be considered as a part of integrated disease management in lentil crops.

**Keywords**: Microwave, Atmospheric non-thermal plasma, lentil, seed-borne, Botrytis grey mould

# 1 Introduction

*Botrytis* grey mould is one of the important seed-borne diseases of pulse crops and vegetables. It can be transmitted from seed to seedling, without any visible symptoms, in many agricultural products such as lentil (Lindbeck et al., 2009), primula (Barnes & Shaw, 2003) and lettuce (Sowley et al., 2010). *Botrytis* grey mould is caused by *Botrytis cinerea* (*B. cinerea*) in lentil and it can reduce the crop yield dramatically, especially in cool and humid seasons. Lentil is one of the important pulse crops and a valuable source of protein, carbohydrate, and fibre. This is known as a cool-season crop in Australia and its yield is threatened by fungal diseases, including *Botrytis* grey mould, which is primarily controlled by fungicides (Lindbeck et al., 2009). There are still concerns about the emergence of resistance in pathogen populations of crops as well as the environmental and health impacts due to the overuse of these chemicals. There have been attempts to find a proper non-chemical method to be considered as part of an integrated disease management strategy in pulse crops, including conventional (Burgess, 1997) and microwave (Taheri et al., 2020; Taheri et al., 2019a) thermal treatment. However, limitations exist with these thermal treatments including the concern of seed quality damage, long exposure times and high energy demands. These limitations have been the motivation for developing a fast and non-thermal physical method for seed treatment.

Among the physical methods of seed treatment, non-thermal plasma has gained an increasing interest in recent years. A considerable amount of literature has been published on the potential of cold plasma for plant growth enhancement (Randeniya, 2015) and removing pathogenic bacteria and storage fungi from the seeds of various crops, including rice (Khamsen et al., 2016), wheat (Thomas-Popo et al., 2019), maize (Zahoranová et al., 2018) and other grains (Selcuk et al., 2008). Although the fungicidal effect of cold plasma has been confirmed for some of the pathogens related to grains (Filatova et al., 2012; Jo et al., 2014; Pérez Pizá et al., 2018; Zahoranová et al., 2016), there is still a lack of information about its effect on a wide range of seed-borne pathogens and the quality of legume seeds.

There are different sources and apparatus for creating a cold plasma for seed treatment. Electromagnetic waves, at a frequency of 2.45 GHz (microwave), is one of the sources, which can be utilised to create the plasma without the need for an electrode. This source has been used to produce plasma processed air (PPA) that has been explored for its antimicrobial activity on fresh produce and seeds (Hertwig et al., 2015; Schnabel et al., 2018) and also to process water, usually called plasma-activated water (PAW), that has been proven to have decontamination (Schnabel et al., 2019) and plant growth enhancement (Kang et al., 2019) effects. Radiofrequency plasma has also been successfully applied to modify wettability and enhance germination of lentil

(Bormashenko et al., 2012) and bean (Bormashenko et al., 2015) seeds at a power of 20 W and a pressure of 0.067 Pa.

Therefore, the objective of this study was to explore the potential of microwave downstream plasma to decontaminate lentil seeds from the seed-borne *Botrytis* pathogen. For this purpose, contaminated seeds were exposed directly to plasma with argon gas or remotely utilising air or a mixture of air and argon as the process gas. Any positive or negative effect of plasma treatments on the seed quality was also investigated.

# 2 Materials and Methods

## 2.1 Lentil seeds preparation

Lentil seeds (type Bolt) were collected from a local farm in Horsham, Victoria, Australia. The 100-seed weight was 4.27±0.09 g, and average diameter and thickness of twenty seeds, measured using a digital calliper, were 5±0.19 and 2.4±0.14 mm, respectively. The initial moisture content of the seeds was determined as 10% (wb) by drying the seeds at 130°C for 20 h. Preparation of *Botrytis* culture and the procedure of inoculation of the seeds with the spores was followed from previously published work from our lab (Taheri et al., 2020; Taheri et al., 2019a). *B. cinerea* (isolate: 174/02) isolated from lentil seeds and provided by South Australian Research and Development Institute (SARDI). The isolate was reproduced by subculturing on potato dextrose agar (PDA) and incubation for 2 weeks at 22 °C, 12 h/12 h dark/light cycle under fluorescent (OSRAM TLD/18W) and Ultraviolet (UV) lights (PHILIPS BLB/18W). The spore solution for inoculation of the seeds was prepared by flooding the plates with sterile distilled water (containing 0.01% tween 20) and gently rubbing the surface of the culture by a glass rod followed by passing through two layers of sterilized cheesecloth. The spore solution was diluted to obtain a concentration of $10^4$-$10^5$ spore ml$^{-1}$ and 25 ml of this solution was used for inoculation of 100 g of lentil seeds.

## 2.2 Plasma system

The plasma system consists of a 3-kW magnetron and three-stub auto-tuner to minimize reflection of the microwave energy. Plasma is created in the downstream plasma source with $TE_{10}$ mode developed by Sairem Co. (WR340 3 XX, Neyron, France). The system works at atmospheric pressure and can be fed by gases of argon and air. The plasma in the quartz tube is cooled down by the water and airflow. A mass flow controller (EL-Flow Prestige, Bronkhorst®, Netherlands) was used to control the gas flow rate and mix the gas flows at an accurate ratio where needed. Forward and reflected microwave powers were monitored using the Auto-tuner (S-Team –

HOMER Automatic Impedance Analyzer and Matching System, Bratislava, Slovak Republic) with HomSoft software (Version 5.0.0.2). The whole schematic of the system is shown in Figure 3.1.

#### *2.2.1 Simulation of electric field and temperature of the treated seeds*

A simulation of the system was performed using the commercial software XFdtd® 7.5.0 (Remcom, Inc., State College, PA) with argon gas at atmospheric pressure as the solid sensor inside the quartz tube. The temperature of the lentil seeds after treatments was measured using an infrared thermal camera (FLIR C2 Education kit) by considering lentils' emissivity as 0.95 (Taheri et al., 2019b).

### 2.3 Design of the experiment

#### *2.3.1 Direct plasma*

In a preliminary experiment, the effect of process parameters including microwave power (300 and 500 W), gas flow (15 and 40 $l_n$ $min^{-1}$ , normal litre per minutes), exposure time (15 and 45 s) and seed moisture content (10% and 16%) on the infected seed percentage (IS%) reduction and seed viability were investigated using direct exposure to argon plasma (2 cm below the end of quartz tube). The plasma treatments were carried out in triplicates and all the quality tests were done at least twice for each treatment replicate. The process parameters were chosen in order not to exceed the temperature of 50°C in the lentil seeds. For direct argon plasma treatments, 2.5 g of contaminated lentil seeds were placed inside a ceramic dish and the seeds were exposed to plasma at a distance of 2 cm below the quartz tube, which is about 20 cm below the entrance of microwave into the plasma source. The container was shaken continuously to make sure all sides of the seeds were treated equally.

#### *2.3.2 Remote plasma treatment (exposure to afterglow) or plasma processed air (PPA)*

To investigate the effect of exposure to the plasma afterglow on the infected seeds, the gas flow of 20 $l_n$ $min^{-1}$ containing air/argon (30/70 v/v) and 100% air were utilised to create plasma. Plasma was ignited with 1.5 kW of microwave power being injected into the plasma source and then the power was reduced to 800 W and the treatments were carried out using the plasma operated with 800 W. For each treatment, 2.5 g of contaminated lentil seeds were placed inside a 500-ml Schott Duran bottle and the bottleneck was adjusted just below the quartz tube from the plasma source. Seed temperature did not exceed room temperature during the exposure times in any of the treatments. After each treatment with specified treatment time (10 and 100 s, as well as 150, 200, 300 and 400 s), the bottle's lid was immediately closed and sealed using aluminium tape to trap the processed gas. This was followed by holding the seeds inside the closed bottle for a specified holding time (0, 5, 15, 30, 60 min and 24 h) at room temperature. After the holding times, the bottle was opened to let the PPA out and then after shaking the seeds into a beaker to make sure there was no trapped process gas in them, they were transferred into the polyethylene Ziplock bags until further examinations. The seeds were shaken two or three times during the holding times to expose both sides of the seeds to the reactive gas species.

## 2.4 Evaluation of the fungicidal effect of plasma

Botrytis grey mould selective media (BSM) was prepared with the following ingredients (g/l): glucose 2; PDA 5; Agar 10; tannic acid 5; Ridomil 0.02; Pentachloronitrobenzene (PCNB) solution with concentration of 0.5% in ethanol 4; Zineb 0.00091; streptomycin sulphate 0.1 (Taheri et al., 2020). A mixture of glucose, PDA and agar was autoclaved and cooled to below 50 °C before adding other ingredients. Direct and remote treatments of the seeds were performed three times and after each treatment by the downstream plasma, 24 lentil seeds per replicate (total of 72 seeds per treatment) were subcultured on BSM and the plates were incubated at 23°C with 12 h/12 h dark/light cycle under fluorescent (OSRAM TLD/18W) and near-ultraviolet (UV) lights (PHILIPS BLB/18W). After four days mycelium growth and the colour change of the BSM to golden brown was examined. Seeds with fungal growth were considered infected and the percentage of infected seeds (IS%-4d) was calculated by dividing the number of infected seeds by the total number of sub-cultured seeds. IS% of the treated seeds was compared with that of the untreated control samples any reduction in percentage was calculated as the difference between the IS% of the control and IS% of the treated sample divided by the IS% of the control sample. The same evaluation was carried out after 7 d of incubation (IS%-7d). Infection degree after 4 d of incubation (ID%-4d) was also calculated based on the degree of fungi growth on the seeds. The degree of growth was considered as 1 (low growth of mycelium on the seeds) and 2 (high growth of the mycelium on and around the seeds on the media) and then ID% was calculated as equation (2):

$$IS\%\text{-}nd = \frac{no.\ of\ infected\ seeds\ at\ day\ n}{total\ no.of\ subcultured\ seeds} \times 100 \tag{1}$$

$$ID\% = \frac{(no.\ of\ infected\ seeds\ with\ degree\ of\ 1{\times}1 + no.\ of\ infected\ seeds\ with\ degree\ of\ 2{\times}2)}{total\ no.\ of\ seeds{\times}2} \times 100 \tag{2}$$

## 2.5 Lentil's quality assessment

### *2.5.1 Seed viability*

The seeds were examined for their viability using the standard germination test by placing 10 healthy-looking (symptomless, without discolouration) seeds per replicate (three replicates) in a 90-cm plate containing No.1 Whatman filter paper and 4 ml of sterilized distilled water. Germinated seeds were counted after 4 d of incubation at room temperature. To evaluate seed vigour, seedling fresh weight was determined after 4 d using a four-digit scale and vigour index was calculated as below:

$$Vigour\ Index = \frac{Seedling\ fresh\ weight\ \times Germination\%}{Number\ of\ seeds} \tag{3}$$

### 2.5.2 Antioxidant enzymes activity of the seedling (POX, CAT, SOD, $H_2O_2$)

Ten of the four-day-old lentil seedlings were accurately weighed using a 4-digit scale. The weighed seedlings were mashed using a pre-chilled pestle and mortar in an ice bath and the enzymes were extracted in 50 mM potassium phosphate buffer with a pH of 6.8 for peroxidase (POX) and catalase (CAT) activities and 100 mM potassium phosphate buffer (pH 7.6) for superoxide dismutase (SOD) followed by centrifugation of the homogenised mixture with 11000g at 4°C for 10 min. The supernatants were then collected in the centrifuged tubes and kept in an ice bath for a maximum of 12 h. The CAT activity of the enzyme extract was measured based on the breakdown of $H_2O_2$ in the presence of the enzyme at 240 nm using a UV/visible spectrophotometer (Maehly, 1954). POX activity of the extract was assayed according to the method described by Maehly (1954). $H_2O_2$ content was measured based on the peroxide mediated oxidation of $Fe^{+2}$ and then the reaction of $Fe^{+3}$ with xylenol orange with an extinction coefficient of 220 $mM^{-1}cm^{-1}$ (Bellincampi, 2000). SOD activity was defined according to the method described by Dhindsa et al. (1981), which is based on the ability of the enzyme for inhibition of the photochemical reduction of Nitro Blue Tetrazolium (NBT) by 50%. Protein contents of the enzyme extracts were determined according to Bradford (1976) using Bradford reagent for 1-1400 μg/ml protein (Sigma-Aldrich, NSW, Australia) by mixing 20 μl of the enzyme extract and 200 $\mu l$ of the reagent in microplate wells followed by measuring their absorbance at 595 nm after resting for 5 min at room temperature against a blank containing phosphate buffer and the reagent. The protein concentrations were decided using the standard curve of Bovine Serum Albumin (BSA) at concentrations of 0.1-1 mg/l.

### 2.5.3 Lentil seeds' colour assessment

Lentil seed's colour was assessed by a Nix™ Pro colour sensor. Seven seeds were arranged in a circle on a black surface, and their colours were scanned, followed by extraction of the CIELAB colour space data ($L^*$: lightness, $a^*$: red/green value, $b^*$: blue/yellow value), using the Nix™ Pro mobile application (v 2.6.4). The overall change in the colours, compared to control samples, was determined by calculation of $\Delta E^*$ as below (Mokrzycki & Tatol, 2011):

$$\Delta E^* = \sqrt{(L^* - L^*_{control})^2 + (a^* - a^*_{control})^2 + (b^* - b^*_{control})^2} \tag{4}$$

According to Mokrzycki and Tatol (2011), clear differences in the colours could be observed by human eyes for $\Delta E^{*} > 3.5$.

#### 2.5.4 Apparent contact angle measurement

The effect of plasma treatments on the wettability of the lentil seed's coat was evaluated by measuring apparent water droplet contact angle using Attension Theta Optical Tensiometer (Biolin Scientific, Frolunda, Sweden) with an accuracy of ±0.1 degrees. A 2.5 µl RO (Reverse osmosis) water was put on each seed and simultaneously the video was captured. The apparent contact angles of right and left sides of the droplet on the seeds were calculated by the method of curve fitting to Young-Laplace equation and recorded with the OneAttension software every 0.03 s up to 10 s. The final picture of the water droplet on the seeds as well as the mean value of the right and left apparent contact angle at the time 0 and 10 s were extracted from the software. The apparent contact angles are an average of three measurements for each treatment.

### 2.6 Statistical analysis

General factorial regression or general linear model (GLM) was used to fit the results followed by the evaluation of the significant effects of the factors and their interactions by ANOVA (analysis of variance). The responses were compared and grouped using Fisher LSD (least significant differences) method with 95% confidence level. All the statistical analysis was performed in Minitab version 18.1 (Minitab Inc., Pennsylvania, USA).

## 3 Results

### 3.1 Electric filed distribution and microwave power

Electric field distribution in the downstream plasma reactor, for microwave power of 800 W, is shown in Figure 4.1. By applying this input power, the maximum electric field was estimated as 56,816 V/m. Forward power, for the set power of 800 W, fluctuated between 400 W and 1200 W. Average forward and reflected microwave power, after the plasma ignition, was calculated to be 660 W and 10.68 W respectively for the 800 W set power, which shows that there is less than 2% of the microwave reflection in the downstream plasma system.

### 3.2 Direct exposure to argon plasma, fungicidal effect, and seed viability

According to the result of the general factorial regression on the results from the preliminary experiment, gas flow rate (p-value=0.01 and F-value=7.27) was the most effective factor in reducing the IS%. Based on these results (results in supplementary data), microwave power (300-500 W) did not affect the results, and since it was challenging to reach a stable plasma with a power of 300 W, microwave power of 400 W was applied in the next step with three levels of argon gas flow (20, 30 and 40 $l_n$ $min^{-1}$) and 25-125 s exposure. The results are illustrated in Figure 4.2 (a-d).

As can be seen in Figure 4.2 (c), IS% (after 4 d of incubation) decreased from 70% to 66%, 56% and 51% after 100 s of exposure and 45%, 37% and 12% after 125 s exposure for gas flow rates of 20, 30 and 40 $l_n$ $min^{-1}$ respectively. Germination and vigour index (Figure 4.2 (a & b)) were not significantly affected up to 100 s, while there was a dramatic reduction in germination from 87.5% to 66.6%, 15% and 0%, at 125 s for gas flow rates of 20, 30 and 40 $l_n$ $min^{-1}$ respectively. The seeds' temperature increased to more than 80°C after 125 s (Figure 4.2 (d)).

## 3.3 Remote treatment of the seeds with afterglow flow of plasma, fungicidal effect, and seed viability

Analysis of IS% using general factorial regression with three factors of treatment time (A), holding time (B) and air% (C) revealed that the three-way interaction (A.B.C) was significant (p-value = 0.041). Therefore, the two-way ANOVA was applied to compare the obtained results for each air% (30% and 100%) separately. Figure 4.3 (a-f) represents the results of lentil seeds treated remotely, which can be called the afterglow of the plasma, using a mixture of 30% air/ 70% argon plasma. Although there were slight increases in the germination and vigour index of healthy seeds (symptomless seeds) after some of the treatments, such as treatment time of 100 s and holding time of 15 and 60 min (an increase from 92.5% to 100% in germination and from 0.1 to 0.12 in vigour index) (Figure 4.3 (a and b)), no statistically significant differences were found among them. IS% after 4 d of incubation (c), ID% after 4 d of incubation (d), and IS% after 7 d of incubation (f) were significantly affected by treatment (p-value of 0.000) and holding times (corresponding p-values of 0.026, 0.015, 0.004). However, increasing the treatment time from 10 s to 100 s was not beneficial in decreasing the IS% or ID%. The best process parameters at this stage were treatment time (t) – holding time (H) of t10s-H60min and t100s-H15min, which reduced the IS%-4d from 88.5% to 40.3% and 36.1% respectively and IS%-7d from 94.4% to 63.9% and 73.6% respectively. Germination of infected seeds on the selective media (Figure 4.3 (e)) increased significantly at t10s-H15min (from 38.9% to 68%) and decreased at t100s-H24h (from 38.9% to 19.4%). Although holding time significantly affected IS% reduction, no significant change was observed by increasing it from 60 min to 24 h.

The results of lentil seed treatments using 100% air plasma are shown in Figure 4.4 (a-f). Here again, no significant changes were observed in germination and vigour index of the healthy-looking seeds, but treatment time and holding time significantly affected IS%-4d, IS%-7d and

ID%-4d. A treatment time of 100 s with the holding time of 5 min reduced IS%-4d from 88.5% to 33.3%, ID%-4d from 67.2% to 19.4% and IS%-7d from 95.8% to 72%. Although no more reduction in IS%-4d and ID%-4d occurred for 100 s treatment by increasing holding time from 5 min to 24 h, a further reduction in IS%-7d to 56.9% was achieved after 24 h of holding time. The results for 10 s treatment time were quite different from those for 30% air plasma. With 10 s treatment time, the results for IS%-4d and ID%-4d were significantly affected by a holding time after 60 min and 24 h (44.8% and 26.4% respectively), while IS%-7d was just significantly reduced after 24 h of holding time (from 95.8% to 73.6%), compared with the control samples. Germination of infected seeds on the selective media (Figure 4.4 (e)) increased significantly with a treatment time of 100 s and holding time of 15 min.

Considering that the treatment time was the most effective factor in the reduction of IS%, treatment time was increased up to 400 s to treat the seeds with 100% air plasma followed by a holding time of 5 min. The results (Figure 4.5 (a-d)) indicated that increasing treatment time from 100 s to 400 s did not reduce IS%-4d to lower than 33%. However, IS%-7d decreased to 58% after 200 s treatment compared to 72% for 100 s treatment time. There was also no more change in the germination of infected seeds on BSM and ID%-4d by increasing the treatment time from 100 s to 400 s (data not shown).

CAT, POX, SOD activities, as well as $H_2O_2$ content of lentil seedlings for effective treatments from the direct and remote mode of exposures, are presented in Table 4.1. There were no significant changes in POX and SOD activities after any of the treatments. However, CAT activity increased slightly through all the treatments and this increase was statistically significant in the seeds treated with 30% air for 10 s and a holding time of 24 h, which increased CAT from 0.8 to 1.1 mM $H_2O_2$ mg$^{-1}$ min$^{-1}$. Correspondingly, $H_2O_2$ content significantly decreased at the treatment time of 10 s and holding time of 24 h for both remote treatments with 30% (from 0.88 to 0.62 $\mu$mole g$^{-1}$) and 100% air (from 0.88 to 0.67 $\mu$mole g$^{-1}$) plasma.

*Table 4.1 Antioxidant enzymes activities of lentil seedlings before and after the treatments with direct and remote plasma[1];*

| Mode of treatment | Air /Argon% | Treatment time (s) | Holding time | CAT (mM $H_2O_2$ mg$^{-1}$ min$^{-1}$) | | POX (mM guaiacol mg$^{-1}$ min$^{-1}$) | | SOD (Unit mg$^{-1}$) | | $H_2O_2$ ($\mu$mole g$^{-1}$) | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Remote | 100/0 | 100 | 5 min | 1.019 ±0.101 | ab | 7.888 ±0.624 | a | 57.82 ±3.26 | a | 0.784 ±0.019 | ab |
| | | 400 | 5 min | 0.938 ±0.084 | abc | 9.148 ±0.669 | a | 55.35 ±6.29 | a | 0.790 ±0.023 | a |
| | | 10 | 24 h | 0.995 ±0.064 | abc | 8.091 ±0.564 | a | 58.32 ±3.72 | a | 0.672 ±0.075 | bc |
| | 30/70 | 100 | 15 min | 0.947 ±0.074 | abc | 8.964 ±0.698 | a | 54.50 ±10.4 | a | 0.901 ±0.022 | a |
| | | 10 | 24 h | 1.106 ±0.060 | a | 7.876 ±0.763 | a | 51.15 ±2.10 | a | 0.627 ±0.020 | c |
| Direct | 0/100 | 100 | - | 0.996 ±0.060 | abc | 9.355 ±0.609 | a | 65.69 ±7.00 | a | 0.832 ±0.050 | a |
| Control | - | - | - | 0.807 ±0.040 | bc | 8.46 ±0.489 | a | 56.69 ±7.01 | a | 0.883 ±0.041 | a |

[1] data are ±SE with n=6; numbers in each column which do not share a letter are significantly different according to Fisher's LSD method with p<0.05.

## 3.4 Effect of the treatments on the lentil seed colour

The results of the CIE Lab and the overall colour change compared to the untreated control samples ($\Delta E^*$) are represented in Table 4.2. Remote treatment of the seeds with 30% air plasma for 10 and 100 s and holding time of 24 h reduced lightness from 51.4 to 48.02 and 43.4 but increased a* from 13.87 to 16.23 and 16.7 and b* from 21.43 to 24.27 and 26.15 respectively. Correspondingly, for 100% air plasma, a holding time of 24 h and treatment times of 10 and 100 s caused a reduction in the L* from 51.4 to 48.02 and 43.42, an increase in a* from 13.87 to 15.08 and 15.67 and an increase in b* from 21.43 to 23.45 and 24.87. Likewise, $\Delta E^*$ dramatically increased to over 4 for 24 h holding time at both treatment times and air percentages. Figure 4.7 illustrates the results of CIE Lab for longer treatment times and 5 min holding time for 100% air plasma. This shows that the same change in colour as 24 h holding time could happen at 400 s treatment time with a reduction in lightness and an increase in redness (a*) and yellowness (b*). Colour change of the lentil seeds treated with 30% air can be observed in Figure 4.8 for the treatment time of 100 s and holding times of 0, 30 min and 24 h.

*Table 4.2 CIE L* a* b* data for untreated and treated lentil seeds with direct and remote plasma at different holding times[1];*

| Mode of treatment | Air /Argon% | Treatment time (s) | Holding time | L* | | a* | | b* | | $\Delta E^*$ |
|---|---|---|---|---|---|---|---|---|---|---|
| Remote | 100/0 | 100 | 0 min | 51.15±0.65 | ab | 13.35±0.64 | e | 21.28±0.54 | efgh | 0.596 |
| | | 100 | 5 min | 50.30±0.91 | ab | 14.63±0.45 | bcde | 22.08±0.43 | defg | 1.487 |
| | | 100 | 24 h | 43.42±2.14 | *d | 15.67±0.61 | abc | 24.87±1.66 | *abc | 8.874 |
| | | 10 | 24 h | 48.02±0.94 | bc | 15.08±0.75 | abcd | 23.45±1.00 | bcde | 4.119 |
| | 30/70 | 100 | 0 min | 50.92±0.89 | ab | 12.98±0.46 | e | 19.83±0.48 | gh | 1.893 |
| | | 100 | 15 min | 48.58±0.58 | b | 14.13±0.62 | cde | 22.87±0.48 | cdef | 3.177 |
| | | 100 | 24 h | 44.53±1.42 | *d | 16.70±0.60 | *a | 26.15±0.99 | *a | 8.803 |
| | | 10 | 24 h | 45.02±0.73 | *cd | 16.23±0.63 | *ab | 24.27±0.43 | *abcd | 7.372 |
| Direct | 0/100 | 100 | - | 51.37±1.24 | ab | 13.06±0.66 | e | 19.32±0.90 | h | 2.260 |
| Control | - | - | - | 51.40±0.64 | a | 13.87±0.29 | de | 21.43±0.40 | fg | |

[1] data are ±SE with n=6; numbers in each column which do not share a letter are significantly different according to Fisher's LSD method with 95% confidence level; numbers with * are significantly different from control according to Dunnett method with p<0.05.

## 3.5 Apparent contact angle

Figure 4.9 shows the water droplet apparent contact angles on the control and treated lentil seeds. The mean apparent contact angle at the beginning was 117±1° in the control samples and 95±13° in the samples treated with 30% air with a treatment time of 10 s and holding time of 24 h (30%Air-t10s-H24h). This was 101±3.6° and 96.6±23° for the seeds treated with 100% air for 100 s and holding times of 5 min and 24 h respectively. After 10 s of capturing, all these values reduced, and it was lowest for 30%Air-t10s-H24h with 73.3±15° compared to 101.6±4.4° for the control samples. The change in the apparent contact angle in the 10 s treatment was the most for 30%Air-t10s-H24h (22° compare to 15° for the rest of the treatments).

# 4 Discussion

In the direct argon plasma treatments, less IS% was observed by increasing gas flow and exposure time. However, the temperature of the seeds increased to more than 60°C after 75 s of exposure time. Therefore, any reduction of the pathogen from the seeds after this point could be the result of the synergistic effect of the heat and plasma. Interestingly, the IS%-4d slightly increased (although with no statistical significance) after treating the seeds with an argon gas flow of 20 $l_n$ $min^{-1}$ and treatment times of 25, 50 and 75 s. It shows that the effect of plasma on growth enhancement of fungi and seeds should be the same, although the dosage and the treatment time might be different. Šimončicová et al. (2018) showed that plasma treatment could induce antioxidant enzyme activities of *Aspergillus flavus* as a result of the hyphae reacting to the stress. Similar effects might have been evident in the *Botrytis* pathogen, which led to their recovery and even more growth later when enough food was provided through media. However, longer treatment times or higher gas flow rates caused enough damage to them to decrease their growth after 4 d of incubation.

The best result of this stage was achieved by treating the lentil seeds utilising a gas flow of 40 $l_n$ $min^{-1}$ for 100 s. This raised the temperature to 64°C (Figure 4.2, (d)) and reduced IS% (4d of incubation) from 70% to 50% (Figure 4.2, (c)) without any deteriorative effect on seed viability (Figure 4.2, (a) and (b)). This reduction in IS% might be due to the contribution of heat, UV and reactive species (mostly excited argon atoms) of the plasma. To avoid the contribution of the heat, the seeds were treated in a pulse mode to keep the temperature below 60°C. In pulse mode treatment, a time of 150 s was provided using 2 cycles of 75 s, 3 cycles of 50 s or 6 cycles of 25 s. Similarly, a time of 250 s was provided using 10 cycles of 25 s with interval cooling to below 45°C (the results in the supplementary data). There was no significant difference among the treatments according to one-way ANOVA with 95% confidence level, but there was a significant reduction in IS% (4d of incubation) from 70% in the control to 52% in the seeds treated with 10 or 6 cycles of 25 s. The results indicated that an increasing number of cycles from 6 to 10, did not have a beneficial effect on the reduction of IS%, though it affected seed viability. Treatment of the seeds with 6 cycles of 25 s slightly increased their germination. In conclusion, pulse mode treatment in direct argon plasma did not have an added value to the continuous treatment with 100 s exposure time regarding the reduction of IS%. However, it was beneficial in keeping the seeds at a lower temperature and protected them from the deteriorative effect of high temperature on the seed viability and possibly other quality attributes. Therefore, a reduction of 20% in IS%-4d was achieved, but there was no difference between IS% of the treated and non-treated seeds after 7 d of incubation, which suggests that pathogen recovered after a longer time of incubation.

In the treatment of the seeds with the afterglow of the plasma (remote treatment), the reactive species from the air (ROS and RNS) are involved in the reduction of the *Botrytis* pathogen, while in the direct plasma treatments, the reactive species are mainly excited argon atoms. The reactive species in the afterglow of air plasma are mainly atomic oxygen (O), atomic nitrogen (N), singlet oxygen ($O_2(a^1\Delta_g)$), exited metastable species of nitrogen ($N_2$(A) and $N_2$(a')) (M Moisan et al., 2014) and the species produced from their reactions such as NO as well as the energy released in the form of UV. In the present study, the air was used as the working plasma gas, which is a combination of about 21% oxygen and 78% of nitrogen. However, Michel Moisan et al. (2013) showed that a combination of $N_2$-$O_2$ with 0.5% of oxygen provided the most UV intensity, which was the result of having the most NO$\gamma$ molecular systems in the afterglow of the microwave plasma. Takamatsu et al. (2014) also indicated that the most NO radical production occurred when a 1:1 ratio of $N_2$: $O_2$ was used. Therefore, the proportions of the reactive species could vary when 100% or 30% with argon was used as the plasma working gas. This could be the reason for the difference in their efficiency in the reduction of IS% at the same treatment or holding times.

Any reduction in IS% during treatment of the seeds with the afterglow of plasma (remote treatments) and without any retention time could be the results of bombardment with the afore-mentioned reactive species from argon and air. However, the pathogen elimination during the holding times (from 5 min to 24 h) could stem from exposure to the long-lived species (such as singlet oxygen ($a^1\Delta_g$)) as well as the result of their reactions. Here, the damage to the pathogen might be due to the exposure to singlet oxygen which led to structural or permanent damage to the spores due to oxidation. The other explanation of pathogen damage during the holding time could be penetration of atomic oxygen and nitrogen into the stack of surface spores, molecular collisions and production of UV radiation and finally damage of the pathogen (M Moisan et al., 2014).

As suggested before, the damage to the spores of the pathogen, caused by the reactive species and their interactions, could be structural or to DNA material. An *in-vitro* (on water agar) investigation of the effect of surface dielectric barrier discharge on the conidia of *B. cinerea* by Ambrico et al. (2020) showed that the plasma caused the erosion of the conidia's cell wall at first and after three minutes of exposure the damage was irreversible. By increasing the exposure time, they proved that the conidia were destroyed completely after 5 minutes. If the damage is structural due to erosion of the spores' cell wall, they might be able to recover, which could be the reason for the difference between the reduction of IS% after 4 and 7 d of incubation. For example, treatment of the seeds by the afterglow of 30% air for 100 s with 15 min holding time caused a 52.4% reduction in IS%-4d (88.5% to 36.1%), while this reduction after 7 d of incubation was about 21% (94.4% to 73.6%). In a previous study, the results of the microwave thermal treatment of the lentil seeds, which were contaminated with *Botrytis* (Taheri et al., 2020), indicated that the proportion of the IS% reduction remained almost the same after 4 and 7 d of incubation, suggesting permanent damage to the pathogen. However, plasma afterglow treatments caused non-permanent damage to some of the seed pathogen spores, which led to their recovery and regrowth later in their incubation time.

No significant reduction in the IS% was observed by increasing treatment time to longer than 200 s or for a holding time over one hour using 100% air as working gas. This could be due to the deep penetration of the *Botrytis* pathogen in the seed coats. The reactive species from the plasma, as well as UV, cannot penetrate deeply into the seed, which made them a very good surface sterilizer. Therefore, apart from the melanin present in the BC's cell wall (Fillinger & Elad, 2016), which makes them resistant to UV radiation, pathogen location in the seeds could also be a limitation to achieve complete eradication. The reason why there was no more decontamination after one hour holding time could also stem from the limited lifetime of the reactive species created by air flow. Schnabel et al. (2015) analysed the reactive species in the plasma processed air using mass spectrometry and indicated that the species, which were mostly $NO^-$ and $NO^-_2$, were stable for 60 min.

The increase in the germination of the seeds on the media (Germination% on BSM), in Figure 4.3 (e) and Figure 4.4 (e), stems from cleaning them from the pathogen and might not necessarily be the result of the stimulating effect of plasma, which has been mentioned in the literature, as an attractive application of the cold plasma. The reason for this conclusion is that we did not find the same growth enhancement in the symptomless seeds (Figure 4.3 (a and b) and Figure 4.4 (a and b)).

As the results of antioxidant enzyme activities showed, the remote plasma treatment and holding time could trigger CAT activity for scavenging hydrogen peroxide which could lead to fortifying the defence system of lentil plants and make them more immune towards contaminations later in the plants' developmental stages. It has been reported that direct or indirect treatment with plasma could decrease antioxidant enzymes activities such as CAT and SOD in wheat (Kučerová et al., 2019) and soybean (DBD, atmospheric pressure) (Pérez Pizá et al., 2018), while an increase in these enzyme activities was observed in sweet basil seeds (RF, vacuum) (Singh et al., 2019), wheat (RF, vacuum air) (Saberi et al., 2018), artichoke (RF, vacuum $N_2$) (Hosseini et al., 2018), soybean (DBD, atmospheric pressure argon) (Zhang et al., 2017) and mung bean (microplasma, atmospheric air) (Zhou et al., 2016). The difference among the results could stem from different plasma devices with different gases and working pressure or different stages of plant growth used to measure the enzyme activity. Surowsky et al. (2016) explained that carbonylation as a result of an oxidative attack in plasma treatments could be the reason for enzyme reduction such as catalase. Carbonylation is an irreversible reaction of producing free carbonyl groups in some amino acids such as arginine, histidine, lysine, proline, threonine, and tryptophan. They also mentioned that Ozone or UV alone could result in enzyme inactivation. However, no enzyme inactivation was observed in the present study, which might be because the seeds temperature did not increase, or the seeds did not go under an oxidative stress due to overdosage of plasma species.

The results of lentil seeds colour measurement proved that prolonged exposure of the seeds to the afterglow of the plasma or a longer holding time could cause a reduction of the lightness (L*) and a shift toward redness (a*) and yellowness (b*). However, treatment of the seeds for 200 s did not cause any change in L* and a*, while it led to an increase in b*. Values of the overall change in colours, $\Delta E^*$, also indicated large changes (more than 4) as a result of prolonged holding time (24 h). This colour change, which mostly occurred during the holding time, could be the result of photodegradation of the lentil seed coat (reaction with polyphenols, fibres or lipids) by the UV produced as a result of the collision of atomic oxygen and nitrogen, as stated before. It could also be the result of oxidation of the seed coat with singlet oxygen (Wypych, 2018) and could be mostly lipid oxidation, which led the seed coat to be more hydrophilic as confirmed by a reduction in the apparent contact angle. An increase in the seed wettability after cold plasma treatments was also reported by other researchers for wheat (Velichko et al., 2019), rice (Khamsen et al., 2016) and soybean (Pérez Pizá et al., 2018). Ji et al. (2018) applied FTIR spectroscopy on the spinach seed coats and showed that lipid molecules of the coat were altered after plasma treatment leading to more surface hydrophilicity. This phenomenon could be one of the reasons for more water uptake and increased germination of the seeds as a result of plasma treatment. Overall, plasma processed air has a good potential to permanently remove the seed-borne pathogen of lentil seeds as far as the seed colour is not of concern. As the seeds in this study are aimed to be used for sowing purpose, the colour change might not be considered as a detrimental effect on their marketability.

## 5 Conclusion

Treatment of lentil seeds by a combination of the afterglow of atmospheric microwave plasma and holding them in the trapped processed gas from 5 min to 24 h could significantly reduce the *Botrytis* infected seeds and consequently increase their germination. A holding time after each treatment times was necessary for the plasma treatments to significantly reduce the infected seeds. The prolonged retention time of 24 h increased redness and yellowness of the lentil seeds but positively triggered the CAT activity and reduced water droplet apparent contact angle on the seed surface. Applying a mixture of 30% air/70% argon or 100% air as the plasma working gas significantly affected the results. At the same holding times, the differences between the results of IS% for short (10 s) and long (100 s) treatment times were significant with 100% air, but they were not significant for the mixture of air/argon, which suggested a difference between the effective reactive species. The results suggest that afterglow of microwave plasma is a promising tool to be implemented in pulse seeds' integrated disease management as a sustainable and environmentally friendly process.

## 6 Acknowledgement

The authors wish to thank A/Prof Kathryn Mumford and Dr April Li from the faculty of Chemical engineering, The University of Melbourne for their assistance with the contact angle test.

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# Figures

Figure 3.1 Downstream cold plasma system

Figure 4.1 (a) Electric field distribution in the downstream plasma reactor with sliding short distance of 12 cm and microwave power of 800 W, the red arrow shows the direction of microwave entry; Images of the thermal camera for (b) indirect, air and (c) direct, argon plasma treatments of lentil seeds.

Figure 4.2 Results of the treatments with 100% argon plasma at a microwave power of 400 W, seed moisture content of 10%, different gas flow rates and exposure times; (a) Germination rate, (b) Vigour index, (c) Infected seed percentage (IS%), (d) Seed temperature after treatments; bars are standard errors of means for n=3.

Figure 4.3 (a) Germination% of the healthy seeds, (b) Vigour index, (c) Infected seeds% after 4 d of incubation, (d) Infected degree% after 4 d of incubation, (e) Germination% of the contaminated seeds on BSM and (f) infected seed% after 7 d of incubation for the lentil seeds exposed to 30% air plasma remotely at different treatment and holding times; error bars are ±SE with n=3; bars which do not share a letter are significantly different according to Fisher's LSD method with $p<0.05$.

Figure 4.4 (a) Germination% of the healthy seeds, (b) Vigour index, (c) Infected seeds% after 4 d of incubation, (d) Infected degree% after 4 d of incubation, (e) Germination% of the contaminated seeds on BSM and (f) infected seed% after 7 d of incubation for the lentil seeds exposed to 100% air plasma remotely at different treatment and holding times; error bars are ±SE with n=3; bars which do not share a letter are significantly different according to Fisher's LSD method with $p<0.05$.

Figure 4.5 (a) Infected seeds% after 4 d of incubation, (b) infected seed% after 7 d of incubation, (c) Germination% of the healthy seeds and (d) Vigour index of the lentil seeds exposed to remote plasma with 100% air as working gas and holding time of 5 min at different treatment times. error bars are ±SE with n=3.

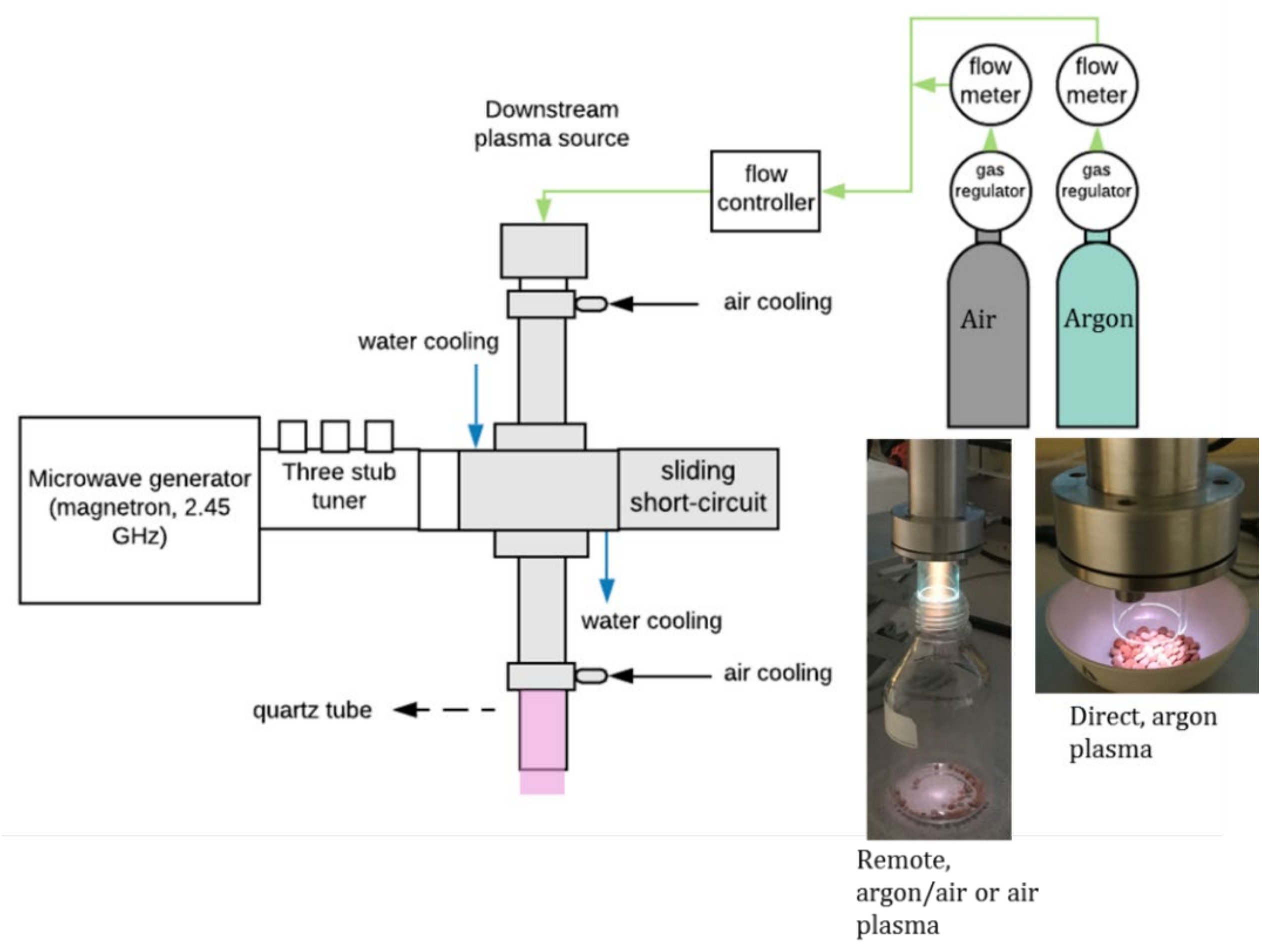
Figure 4.6 Growth of BGM of lentil seeds on Botrytis selective media (BSM), non-treated and treated with remote air plasma for 100 and 200 s and holding time of 5 min.

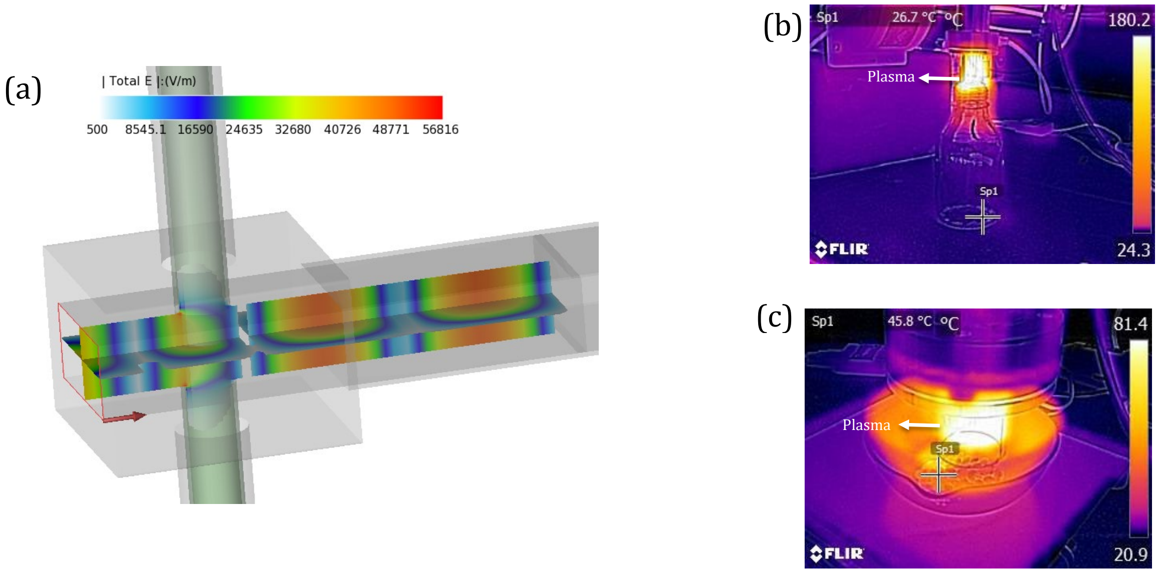
Figure 4.7 L* a* b* data of the lentil seeds exposed to remote plasma with 100% air as working gas at different treatment and holding times; ΔE^* for 200s and 400 are 2.6 and 5.4 respectively;

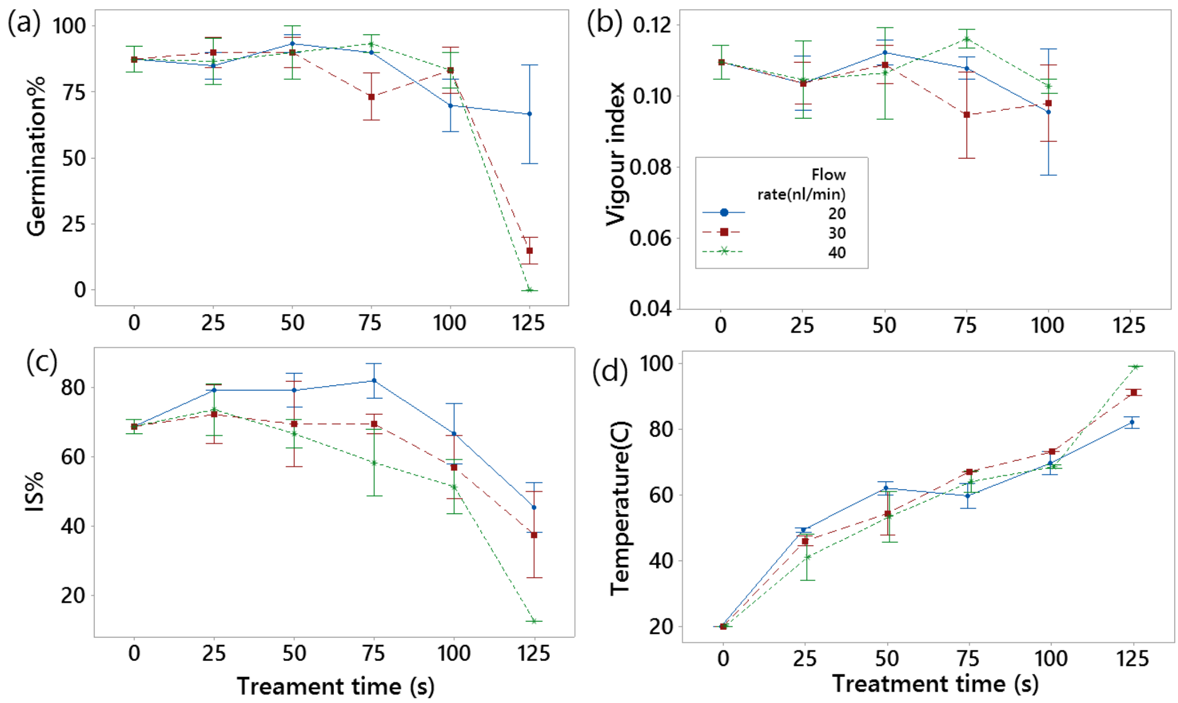
error bars are ±SE with n=6; data with * are significantly different from control according to Fisher's LSD method with $p<0.05$.

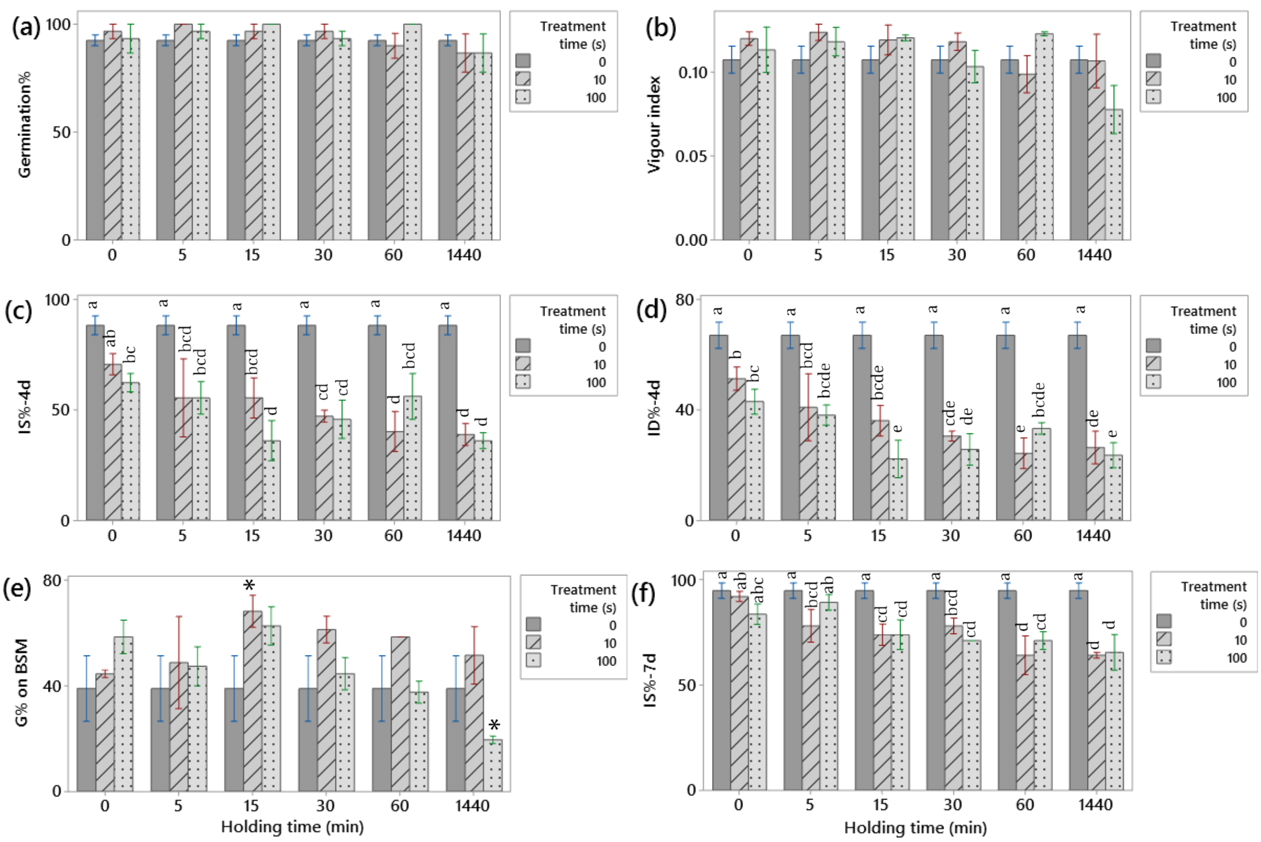
Figure 4.8 Colour change of lentil seeds treated by remote plasma with 30% air for 100s at different holding times.

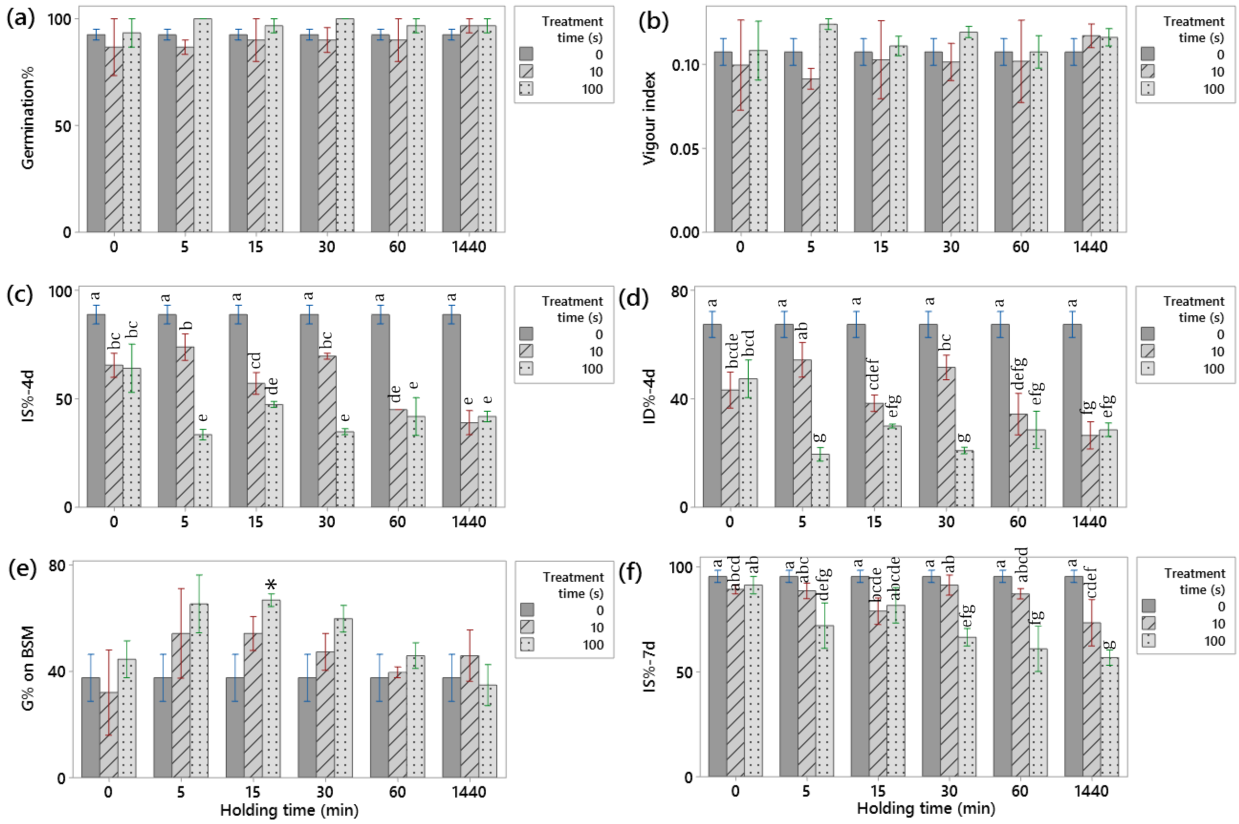
Figure 4.9 (a) Mean Apparent Contact Angle (ACA) of the water droplet (at time 0 and 10 s) and their difference (delta mean ACA) on the control and treated seeds with 100% air, treatment time of 100 s and holding time of 5 min (100% Air-t100s-H5min) and holding time of 24 h (100% Air-t100s-H24h), and with 30% air, treatment time of 10 s and holding time of 24 h (30% Air-t10s-H24h), error bars are standard deviations with n=3, and the bars with * are significantly different from control according to the Fisher LSD method with $p<0.05$; the image of contact angle measurement of (b) control and (c) sample treated with 30% air, treatment time of 10 s and holding time of 24 h.

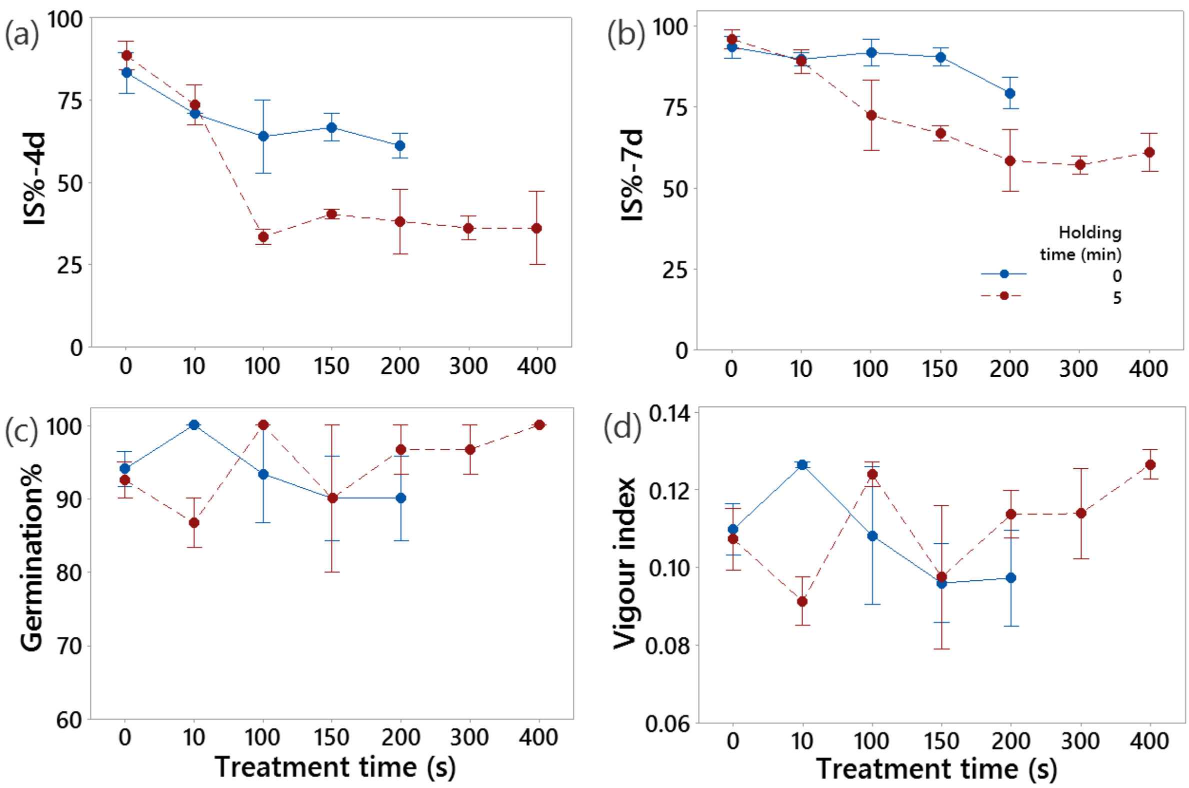

flow meter
flow meter
gas regulator
gas regulator
Air
Argon
Downstream plasma source
flow controller
air cooling
water cooling
Microwave generator (magnetron, 2.45 GHz)
Three stub tuner
sliding short-circuit
water cooling
air cooling
quartz tube
Direct, argon plasma
Remote, argon/air or air plasma

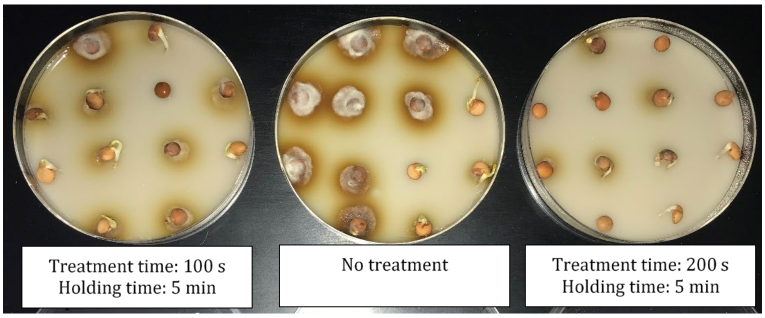

(a)
| Total E |:(V/m)
500 8545.1 16590 24635 32680 40726 48771 56816
(b)
Sp1
26.7 °C
180.2
Plasma
Sp1
FLIR
24.3
(c)
Sp1
45.8 °C
81.4
Plasma
Sp1
FLIR
20.9

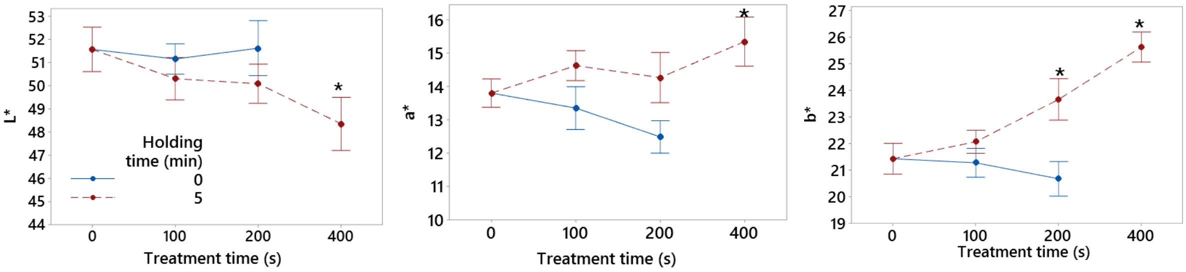

(a)
Germination%
(b)
Vigour index
Flow rate(nl/min)
20
30
40
(c)
IS%
Treament time (s)
(d)
Temperature(C)
Treatment time (s)

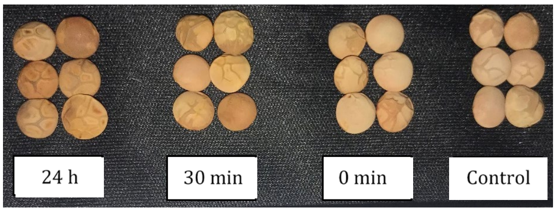

(a)
Germination%
Treatment time (s)
0
10
100
(b)
Vigour index
(c)
IS%-4d
(d)
ID%-4d
(e)
G% on BSM
Holding time (min)
(f)
IS%-7d
Holding time (min)

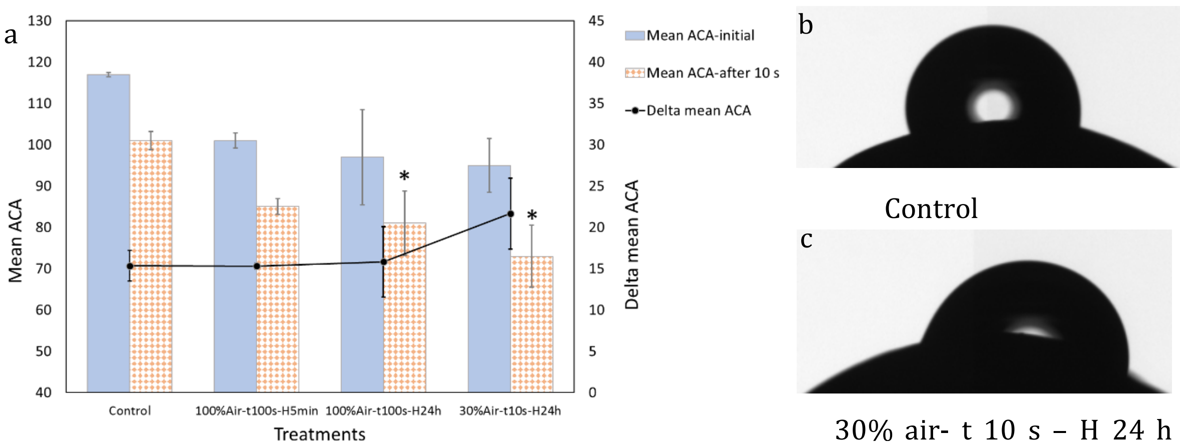

(a)
Germination%
(b)
Vigour index
(c)
IS%-4d
(d)
ID%-4d
(e)
G% on BSM
(f)
IS%-7d
Treatment time (s)
0
10
100
Holding time (min)
0
5
15
30
60
1440


(a)
IS%-4d
(b)
IS%-7d
Holding time (min)
0
5
(c)
Germination%
(d)
Vigour index
Treatment time (s)
0
10
100
150
200
300
400


Treatment time: 100 s
Holding time: 5 min
No treatment
Treatment time: 200 s
Holding time: 5 min


L*
a*
b*
Holding time (min)
0
5
Treatment time (s)
0
100
200
400


24 h
30 min
0 min
Control


a
b
c
Mean ACA
Delta mean ACA
Mean ACA-initial
Mean ACA-after 10 s
Delta mean ACA
Control
100%Air-t100s-H5min
100%Air-t100s-H24h
30%Air-t10s-H24h
Treatments
Control
30% air- t 10 s – H 24 h