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Original Article

# Inactivation of isolated fungi on *Erythrina velutina* Willd. seeds through atmospheric plasma

## Inativação de fungos isolados em sementes de *Erythrina velutina* Willd. por meio do plasma atmosférico

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**Abstract**

This study aimed to evaluate the effect of atmospheric plasma application on the inactivation of fungi on the surface of *Erythrina velutina* seeds and on isolated fungal colonies. Two experiments were conducted using a completely randomized design. First, plasma was applied to the surface of the seeds using helium gas and atmospheric plasma for 3, 6, and 9 min in addition to the control (untreated seeds), constituting seven treatments with five repetitions each. In the second experiment, Petri dishes containing the inoculum of different fungi were treated with atmospheric air plasma for 3, 6, and 9 min (Air-3, Air-6, and Air-9) and were compared with untreated fungi in Petri dishes without treatment (control), totaling four treatments and five repetitions each. We found that the application of atmospheric air plasma to *E. velutina* seeds for 9 min had an antimicrobial effect on the fungi *Aspergillus niger*, *Aspergillus flavus*, *Fusarium* sp., *Brachysporium* sp., and *Rhizopus* sp. The formation of fungal colonies isolated from *E. velutina* seeds was also inhibited by 3 min of exposure to atmospheric air plasma, except for *A. niger*, whose inhibition occurred after 6 min of exposure to atmospheric plasma.

**Keywords:** *Aspergillus* sp., *Brachysporium* sp., *Fusarium* sp., *Rhizopus* sp., seed pathology.

**Resumo**

Este trabalho teve como objetivo avaliar o efeito da aplicação de plasma atmosférico na inativação de fungos na superfície de sementes de *Erythrina velutina* e em colônias fúngicas isoladas. Dois experimentos foram realizados em delineamento inteiramente casualizado: no primeiro, o plasma foi aplicado na superfície das sementes usando gás hélio e plasma atmosférico por três, seis e nove minutos, além do controle (sementes sem tratamento), constituindo sete tratamentos com cinco repetições cada; no segundo experimento, placas de Petri contendo o inóculo de diferentes fungos foram tratadas com plasma atmosférico por três, seis e nove minutos (Air-3, Air-6 e Air-9) e comparadas com fungos não tratados em placas de Petri sem tratamento (controle), totalizando quatro tratamentos e cinco repetições cada. Descobrimos que a aplicação de plasma atmosférico nas sementes de *E. velutina* por nove minutos teve efeito antimicrobiano sobre os fungos *Aspergillus niger*, *Aspergillus flavus*, *Fusarium* sp., *Brachysporium* sp. e *Rhizopus* sp. A formação de colônias fúngicas isoladas de sementes de *E. velutina* também foi inibida por três minutos de exposição à aplicação de plasma atmosférico, exceto para *A. niger*, cuja inibição ocorreu a partir de 6 minutos de exposição à aplicação de plasma atmosférico.

**Palavras-chave:** *Aspergillus* sp., *Brachysporium* sp., *Fusarium* sp., *Rhizopus* sp., patologia de sementes.

## 1. Introduction

*Erythrina velutina* is a species of the Fabaceae family that occurs in the Caatinga biome of Brazil and has a high resistance to drought and vigorous, rapid growth. In this biome, *E. velutina* is popularly known as "mulungu", "corticeira", and "sananduva" and is used for various purposes such as hedge plantings, recovery of degraded areas, landscaping, handicrafts, and production of medicinal products (Ribeiro et al., 2014). Seedlings of this species are produced from seeds that undergo coat dormancy and require pre-germinative treatments to reduce unevenness and accelerate the germination process (Santos et al., 2014).

Contamination by microorganisms may lead to the loss of seed lots that do not reach satisfactory levels of germination, reducing the supply of seeds in the market. This fact is even more worrying for the seeds of forest species because, their seasonal production may be sporadic. Thus, it is necessary to understand the pathogenic agents

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affecting these seeds and the methods that can be used to control these pathogens (Brasil, 2009).

Despite the importance of *E. velutina*, several problems limit the production of its seedlings in large scale, including seed contamination by fungi during storage. The presence of fungi can reduce the capacity for seed germination and increase the number of abnormal seedlings, making the interpretation of germination tests difficult (Selcuk et al., 2008).

Among the measures used to control fungi in seeds, chemical control is the most common. However, it poses risks of environmental contamination, is toxic to humans and animals, is costly, and introduces problems associated with pathogen resistance (Trebbi et al., 2007). Thus, the use of clean technology such as the application of atmospheric plasma has been widely studied in modern agriculture. This technique can replace the chemical treatments used for stored seeds, and its technology is an innovative way to improve the quality and yield of crops (Starek-Wójcicka et al., 2020).

Plasma application utilizes a potential difference between two electrodes immersed in gas, which generates chemical elements such as ions, electrons, neutral particles, energetic particles, and radicals, as well as ultraviolet radiation. These elements inactivate microorganisms through antioxidation, causing cell damage and death (von Woedtke et al., 2013).

The mechanisms underlying the plasma-induced inactivation of microorganisms are not fully understood because of their complex and dynamic nature; however, they can be divided into biological and physical components. According to Liao et al. (2017), electrostatic interruption and oxidation of cellular constituents are the main mechanisms driving the inactivation of microorganisms.

For electrostatic interruption, Lunov et al. (2015) indicated that the charged species generated by plasma accumulated in the cell membrane and produced electrostatic forces that exceeded the tensile force of the membrane, causing it to rupture, followed by cell death. According to the same authors, the oxidation of cellular constituents occurs because of the action of reactive oxygen and nitrogen species, which interact with macromolecules such as lipids, proteins, and DNA, compromising their function and subsequently causing cell death. Reactive oxidative species interact mainly with lipids because of their greater susceptibility and location near the cell surface (Liao et al., 2017). This process begins in the cell membrane, with lipid peroxidation irreversibly compromising the structural integrity of the membrane, damaging DNA and proteins and consequently leading to lysis. Although less studied, electroporation is an important mechanism in the inactivation of microorganisms. It involves the formation of pores in cell membranes by a high-voltage electric field pulse, capable of destabilizing proteins and the lipid bilayer, leading to cell death.

Recently, plasma application studies for the control of fungi in seeds have been carried out for many species of agricultural importance. In hazelnut seeds (*Corylus avellana*), plasma application for 2 min eliminated the spores of *Aspergillus flavus* and *Aspergillus parasiticus* (Dasan et al., 2016). The use of plasma (e.g., 10 kHz, 8 kV pulses for 3 min) in the artificially applied *Escherichia coli* microbiota revealed higher maximum inactivation efficiency for *E. coli* (Butscher et al., 2016), and plasma treatment potentially reduced the microbial load of rapeseed and promoted seed germination and seedling growth rates (Puligundla et al., 2017).

Thus, this study aimed to evaluate the effect of atmospheric plasma application on fungi present on the surface of *E. velutina* seeds and in isolated fungal colonies.

## 2. Material and Methods

The experiment was carried out in a biochemical oxygen demand (BOD) chamber at 26 ± 2°C in the dark at the laboratories of microbiology and phytopathology of the Federal Rural University of the Semi-Arid Region (UFERSA). The experiments were divided into two steps: in the first step, plasma was directly applied to the surface of seeds, and, in the second step, plasma was directly applied to the fungal colonies isolated from the seeds.

*E. velutina* seeds were collected from 20 adult trees located in the Padre Alfredo settlement, Crateús, CE, Brazil (05° 10' 42" S, 40° 40' 39" W, 274 m) and were manually processed to remove any seeds damaged by insects, empty, or cracked. After manual processing of the seeds, they were placed in a sealed glass container and stored for 6 months in a cold room with controlled temperature and relative humidity.

### *2.1. Experiment I*

Five replicates were used for each treatment, represented by five Petri dishes with 10 seeds each. Initially, Petri dishes and potato dextrose agar (PDA) culture medium were autoclaved at 120°C for 30 min. Treatments were applied to the seeds, consisting of control (untreated seeds), helium gas for 3 (He-3), 6 (He-6), and 9 (He-9) min, and air atmospheric plasma applied for 3 (Ar-3), 6 (Ar-6), and 9 (Ar-9) min. After application of the treatments, the seeds were placed in Petri dishes (20 cm diameter) containing PDA culture medium.

Custom plasma equipment was used to apply atmospheric plasma jets using dielectric barrier discharge (DBD). Using a high-voltage DC source, 9 kV pulses were repeated at a frequency of 640 Hz and applied between the two electrodes. An open Petri dish (20 cm in diameter) was placed between the two electrodes (Figure 1). The electric discharge was generated in a flow of 1 L/min (high-purity helium, 99.999%, or atmospheric air) introduced into the Petri dish.

The Petri dishes containing the seeds treated with plasma were closed and kept in a biochemical oxygen demand (BOD) chamber for 7 days at 26 ± 2°C in the dark to allow the development of fungi that were not eliminated during treatments. The incidence of fungi was evaluated by counting the fungal growth visible to the naked eye, and their classification was performed using stereoscopic and optical microscopes and an identification key (Barnett and Hunter, 1998).

A completely randomized design was used, with seven treatments and five replicates (with 10 seeds each). Mean

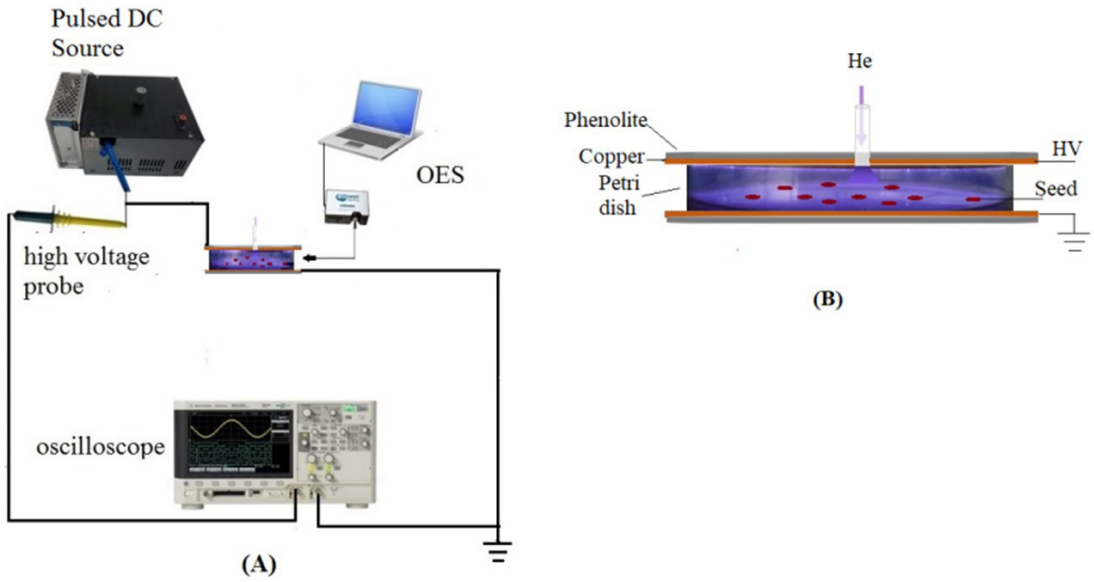


**Figure 1.** Experimental apparatus to treat *Erythrina velutina* seeds using atmospheric dielectric barrier discharge (DBD) plasma. Pulsed DC Source—pulsating direct current source OES—optical emission spectroscopy, He—Helium, and HV—high voltage.

percentages of fungal incidence were compared using ANOVA and Tukey's test at $\alpha = 0.05$, using the statistical program Assistat, beta version 7.6 (Silva and Azevedo, 2009).

*2.2. Experiment II*

To obtain fungal isolates, *E. velutina* seeds not treated with plasma were placed on PDA culture medium on Petri dishes and maintained in a BOD chamber for 7 days at 26 ± 2°C in the dark to favor fungal development. Fungal isolates were purified using subculturing techniques, preserved by the Castellani method (Figueiredo, 1967), and kept in a refrigerated chamber at 5 ± 2°C.

To obtain the inoculum, 7-mm discs of PDA culture medium containing mycelial growth of each fungal isolate were removed from Castellani tubes and transferred to the center of Petri dishes containing PDA culture medium. They were kept in a BOD chamber for 7 days at 26 ± 2°C in the dark to favor the development of fungal isolates.

After 7 days of fungal colony development, the inoculum suspension was prepared by adding 10 mL of sterilized distilled water containing 0.1 mL of Tween 20 to each Petri dish, followed by surface scraping with a flame-sterilized Drigalski spatula. The obtained suspension was filtered through a double layer of sterile gauze to obtain only conidia (Deuteromycota) and oospores (Zygomycota) and subjected to serial dilution and plating to facilitate counting (from 30 to 300 cfu $mL^{-1}$). Then, this dilution (0.1 mL) was evenly distributed using a flame-sterilized Drigalski spatula on the surface of a Petri dish containing PDA culture medium.

Petri dishes containing the inoculum of each fungus obtained from *E. velutina* seeds were subjected to atmospheric air plasma treatment for 3, 6, and 9 min and were compared with controls (CFU). The best treatments in experiment I were chosen, that is, the treatments that most reduced the incidence of fungi in the seeds, and these treatments were applied to the isolates to verify their effectiveness.

The experimental design was completely randomized with four treatments (control, Air-3, Air-6, and Air-9) and five replicates, with each replicate consisting of one Petri dish. Plasma was applied in the same manner as that used for the seed treatments (9 kV voltage and 640 kHz frequency), as indicated in Figure 1. Plasma was chemically characterized using optical emission spectroscopy (OES). Plasma optical spectra were obtained by optical fiber (0.6-mm diameter) positioned laterally to the jet and transmitted to an optical emission spectrometer (range 200–700 nm) to diagnose plasma species. The main peaks resulted from the excitation of the nitrogen ($N_2$) molecules present in the air, all from the second positive system of $N_2$, with different energetic levels. Peaks from the first positive system were also observed: $N_2^+$, apart from OH, and O. The most intense peaks occurred at 337 nm, 357 nm, and 380 nm, corresponding to the transitions (v'v") of (0'0"), (0'1"), and (0'2"), respectively.

After applying the treatments, the Petri dishes were closed and maintained in a BOD chamber at 26 ± 2°C in the dark and monitored daily for 3-7 days, depending on the fungal isolate, until colonies appeared. Fungal colonies were counted and calculated as CFU per mL (cfu $mL^{-1}$) in the initial suspension.

The mean numbers of cfu $mL^{-1}$ were compared using ANOVA and Tukey's tests at $\alpha = 0.05$, using the statistical program Assistat, beta version 7.6.

## 3. Results

### 3.1. Experiment I

The study on the presence of fungi in *E. velutina* seeds showed a high fungal incidence (98%) in the seeds of the control treatment (Table 1). Treatment with dielectric barrier discharge plasma with helium gas for 3, 6, and 9 min was not effective ($p < 0.05$) in reducing the presence of fungi compared to the control treatment, with an incidence of 94–98% (Table 1). The treatments with dielectric plasma of barrier discharge with atmospheric gas for 3 and 6 min were also not significantly effective ($p < 0.05$) in reducing the presence of fungi compared to the control treatment, with an incidence from 96% to 98%. However, treatment with dielectric barrier discharge plasma with atmospheric gas for 9 min was significantly ($p < 0.05$) effective in reducing the presence of fungi in *E. velutina* seeds, reducing the presence of fungi compared to the control treatment by 18.4% (Table 1).

**Table 1.** Presence of fungi in *Erythrina velutina* seeds after treatment with dielectric barrier discharge plasma, applied for three, six and nine minutes with helium gas (He-3, He-6 and He-9) and atmospheric air (Air-3, Air-6 and Air-9).

| Treatment | Presence of fungi (%) |
|---|---|
| Control | 98 a |
| He-3 | 98 a |
| He-6 | 94 a |
| He-9 | 94 a |
| Air-3 | 98a |
| Air-6 | 96 a |
| Air-9 | 80 b |
| C.V. (%) | 6.71 |

Means followed by the same letter do not differ by the Tukey test, at 5% probability. C.V. = coefficient of variation.

Figure 2 shows the incidence of fungi in *E. velutina* seeds in the control treatment and after treatment with dielectric barrier discharge plasma applied for 3, 6, and 9 min with helium gas (He-3, He-6, and He-9) and atmospheric air (Ar-3, Ar-6, and Ar-9).

Studies on the incidence of fungi on *E. velutina* seeds have highlighted *A. niger*, *A. flavus*, *Fusarium* sp., *Brachysporium* sp., and *Rhizopus* sp. as major fungal agents (Table 2). In the control, the incidence of fungi was 68%, 18%, 6%, 2%, and 6% for *A. niger*, *A. flavus*, *Fusarium* sp., *Brachysporium* sp., and *Rhizopus* sp., respectively. In the He-3, He-6, Ar-3, and Ar-6 treatments, incidences of 60%, 50%, 66%, and 20% were observed for *A. niger*, respectively; 14%, 4%, 4%, and 8% were observed for *A. flavus*, respectively; 2%, 2%, 4%, and 8% were observed for *Fusarium* sp., respectively; 2%, 20%, 10%, and 14% were observed for *Brachysporium* sp., respectively; and 10%, 8%, 10%, and 12% were observed for *Rhizopus* sp., respectively. In the seeds treated with He-9, *Rhizopus* sp. was not observed, while incidences of 50%, 26%, 2%, and 6% for *A. niger*, *A. flavus*, *Fusarium* sp., and *Brachysporium* sp., respectively, were observed. In the Air-9 treatment, *Brachysporium* sp. and *Rhizopus* sp. were not observed; only fungi *A. niger* (42%), *A. flavus* (10%), and *Fusarium* sp. (8%) were observed (Table 2).

### 3.2. Experiment II

The application of treatments of discharge plasma by dielectric barrier from atmospheric gases Ar-3, Ar-6, and Ar-9 reduced the CFU of fungi isolated from *E. velutina* seeds. Reductions of 12.9%, 54.8%, and 67.7% were observed for *A. niger*, respectively; reductions of 54.2%, 70.8%, and 88.5% were observed for *A. flavus*, respectively; and reductions of 60%, 85%, and 92.5% were observed for *Brachysporium*

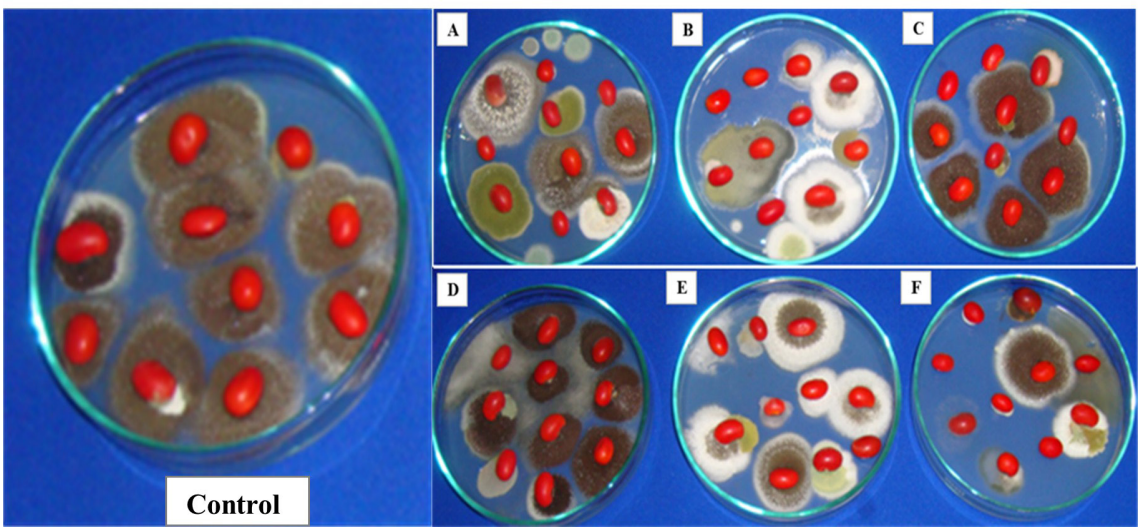


**Figure 2.** Fungal mycelial growth in *Erythrina velutina* seeds after treatments with dielectric barrier discharge plasma. A, B and C = application of helium gas for three, six and nine minutes, respectively; D, E and F = application of atmospheric air for three, six and nine minutes, respectively.

**Table 2.** Incidence of fungi in *Erythrina velutina* seeds treated with dielectric barrier discharge plasma, generated by helium gas (He) and atmospheric air (Air) applied for three, six and nine minutes.

| Treatment | Incidence of fungi in *Erythrina velutina* seeds (%) | | | | |
|---|---|---|---|---|---|
| | ***A. niger*** | ***A. flavus*** | ***Fusarium* sp.** | ***Brachysporium sp.*** | ***Rhizopus* sp.** |
| Control | 68 | 18 | 6 | 2 | 6 |
| He-3 | 60 | 14 | 2 | 2 | 10 |
| He-6 | 50 | 4 | 2 | 20 | 8 |
| He-9 | 50 | 26 | 2 | 6 | - |
| Air-3 | 66 | 4 | 4 | 10 | 10 |
| Air-6 | 20 | 8 | 8 | 14 | 12 |
| Air-9 | 42 | 10 | 8 | - | - |

sp., respectively, compared to the control treatment. The application of plasma from atmospheric gases Ar-3 and Ar-6 reduced the CFU of *Fusarium* sp. by 38.1% and 76.2%, respectively, and *Rhizopus* sp. by 66.7% and 80%, respectively, compared to the control (Figure 3).

The effects of all treatments on the inhibition of fungal mycelia are shown in Figure 4.

Figure 5 illustrates the optical emission spectra of atmospheric air plasma. OES was used to determine the nature of the excited species produced in the discharge layer and to evaluate the rotational and vibrational temperatures of the plasma. Using this technique, two peaks of the reactive species of oxygen and $N_2$, especially hydroxyls, ozone, oxygen, and nitrogen radicals, were observed.

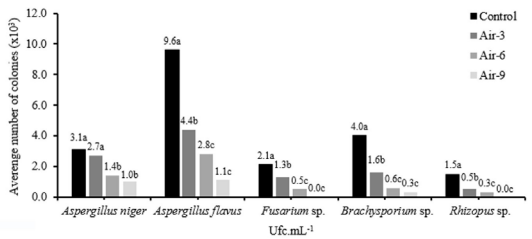


**Figure 3.** Colony-forming units per mL (cfu.mL$^{-1}$) of fungi isolated from *Erythrina velutina* seeds, after treatment with dielectric barrier discharge plasma generated by atmospheric air applied for three, six and nine minutes (Air-3), (Air-6) and (Air-9), respectively. Letters compare plasma treatment for each fungus isolated by Tukey test at 5% probability.

## 4. Discussion

### *4.1. Experiment I*

Treatment of seeds with atmospheric air plasma for 9 min led to significantly ($p < 0.05$) lower fungal growth, differing from the other treatments and the control. Inactivation of microorganisms by plasma may occur because of the disruption of cell membranes by electrons, UV radiation, and/or suffocation by ozone (Tiwari et al., 2010), which can cause damage and cell death (von Woedtke et al., 2013).

The results of this study suggested that ozone had a greater effect on fungal inactivation because ozone was the main difference between the types of atmospheres used. Although there was no statistically significant difference, fungal growth tended to decrease with treatment time. Nishime et al. (2017) found that a plasma jet at atmospheric pressure proved to be efficient in reducing the growth of three species of microorganisms (*Enterococcus faecalis*, *Pseudomonas aeruginosa*, and *Candida albicans*) when applied for 150 and 180 s. According to these authors, ozone gas exposure times were the most effective at inactivating microorganisms.

Atmospheric air plasma releases large amounts of ozone and has proven to be efficient in controlling fungi in stored grains (Pereira et al. 2008). Application of this gas for 60 min also reduced the populations of *Alternaria* sp., *Fusarium* sp., *Aspergillus* sp., and *Penicillium* sp. in sunflower seeds (*Helianthus annuus* L.) (Rodrigues et al., 2015). Phytopathogens normally associated with seeds can adhere to the surface, the most superficial layers of the endosperm, or the embryo (Brasil, 2009). Thus, treatments that reach only the surface may not be effective in eliminating pathogens, as in the treatment with DBD plasma, which acts up to a few nanometers (Keen et al., 2006).

The fungi found in seeds following plasma treatment were *A. niger*, *A. flavus*, *Fusarium* sp., *Brachysporium* sp., and *Rhizopus* sp. Seeds treated with atmospheric plasma for 6 min (Ar-6) presented a lower incidence of *A. niger* (20%) than that of the other treatments. After the same 6-min period, the incidence of *A. flavus* and *Fusarium* sp. with the application of helium gas was 4% and 2%, respectively. There was no incidence of *Brachysporium* sp. and *Rhizopus* sp. after the application of atmospheric plasma for 9 min. The effectiveness of plasma for inactivation of microorganisms also depends on biological factors such as species, infestation level, and developmental stage (Misra et al., 2011).

Kordas et al. (2015) also found a reduction in the number of propagules of the fungi *Aspergillus*, *Fusarium,* and *Rhizopus* in wheat seeds treated with plasma for

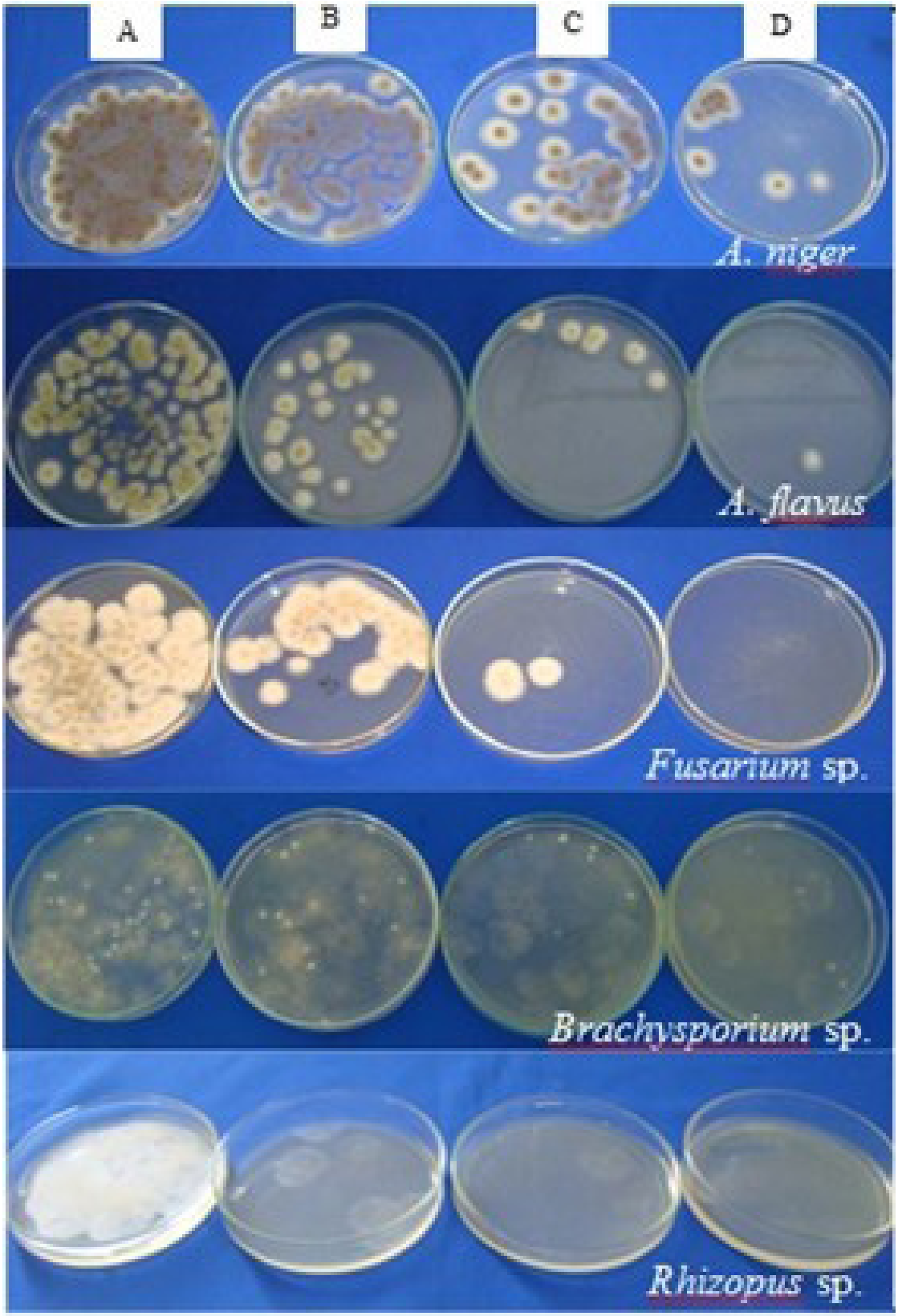


**Figure 4.** Number of fungal mycelia isolated from *Erythrina velutina* seeds after treatments with atmospheric air plasma. A = Control; B = 3 min.; C = 6 min.; D = 9 min.

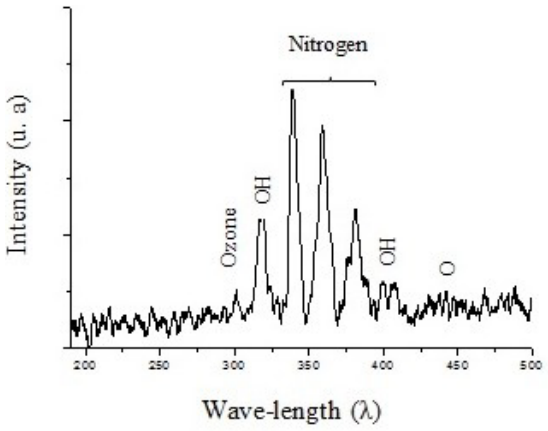


**Figure 5.** Optical Emission Spectroscopy of atmospheric air plasma.

10 s. According to the authors, this reduction was more pronounced in the two latter genera.

Plasma application is a promising alternative for the elimination of several microorganisms (Nishime et al., 2017). Different types and combinations of gases can be used to further increase the application of plasma, but atmospheric air can be a practical and economical option, as it has proven to be effective for this purpose. The application of atmospheric air plasma for 15 min reduced the presence of *Aspergillus* sp. (cfu $g^{-1}$) on the surface of wheat (*Triticum* sp.), rye (*Secale cereale*), corn (*Zea mays*), and chickpea (*Cicer arietinum*) seeds (Selcuk et al., 2008).

*4.2. Experiment II*

In the present study, the application of plasma to fungal conidia and oospores reduced CFU for all the fungi evaluated. The application of atmospheric air plasma for 6 (Air-6) and 9 (Air-9) min led to a greater reduction in the CFU of *A. niger*, differing significantly from the control and from the 3-min treatment (Air-3). The CFU of the fungi *A. flavus* and *Brachysporium* sp. decreased with increasing exposure to plasma, differing significantly from the Air-3 treatment. *Fusarium* sp. and *Rhizopus* sp. were more effectively controlled, as these fungi were completely eliminated by Air-9 treatment.

Significant reductions in *Aspergillus* sp. infestation on the surface of seeds of wheat, barley, oak, lentil, rye, corn, and chickpea using atmospheric air plasma were found by Selcuk et al. (2008). Plasma is a partially ionized gas containing molecules, electrons, ions, atoms, and free radicals that can act as inactivators for a wide range of microorganisms, including spores and conidia. Various types of ions, radicals, electrons, and ultraviolet rays present in plasma contribute to its antimicrobial effects (Shintani et al., 2010). Atmospheric plasma technology has proven to be effective in reducing the formation of *Fusarium oxysporum* spores after 1 min of treatment in *Oryza* sp. seeds (Jo et al., 2014). In hazelnut seeds (*Corylus avellana*), plasma application for 2 min eliminated the spores of *A. flavus* and *A. parasiticus* (Dasan et al., 2016).

In the optical emission spectrum of the plasma, two peaks of reactive oxygen and $N_2$ species were observed, especially hydroxyls, ozone, and radicals of oxygen and nitrogen. According to the literature, gases such as ozone, OH radicals, electrons, and UV radiation are the main inactivators of fungi (Scholtz et al., 2015).

The efficiency of non-thermal plasma in controlling microorganisms has been observed in different studies such as those of Selcuk et al. (2008), with *Aspergillus* and *Penicillum*, Na et al. (2013), with *Neurospora crassa*, *Fusarium graminearum,* and *F. oxysporum*; Jo et al. (2014), in the reduction of the formation of *Gibberella fujikuroi* colonies; by Panngom et al. (2014), in the inactivation of *F. oxysporum*; by Kim et al. (2011), with *Listeria monocytogenes*, *E. coli,* and *Salmonella typhimurium*; and by Jie et al. (2015), in the reduction of microorganisms present in water. Thus, the use and enhancement of this technology is very promising for the elimination of microorganisms without damaging the environment.

In summary, the application of atmospheric air plasma to *E. velutina* seeds for 9 min has an antimicrobial effect on the fungi *A. niger*, *A. flavus*, *Fusarium* sp., *Brachysporium* sp., and *Rhizopus* sp. The formation of colonies of fungi isolated from *E. velutina* seeds was inhibited by the application of atmospheric air plasma for 3 min, except for *A. niger*, whose inhibition occurred after 6 min of exposure to atmospheric plasma.

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