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Application of Cold Atmospheric Pressure Plasma Jet Results in Achievement of Universal Antibacterial Properties on Various Plant Seeds

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Abstract: In view of a constant growth in the human population on Earth, the provision of a necessary amount of high-quality food looks challenging. As over 10% of the crop yields are annually lost due to the presence of phytopathogens, the development of novel, eco-friendly methods of pest eradication might contribute to avoiding nutritional shortages. Here, we propose a controlled application of cold atmospheric pressure plasma (CAPP) generated in the form of an atmospheric pressure plasma jet (APPJ), for which we conducted multivariate optimization of the working parameters with the use of the design of experiments (DoE) in addition to the response surface methodology (RSM). After estimating the optimal operating conditions of APPJ, we determined the inactivation rates caused by 2 min CAPP exposure towards bacterial phytopathogens from three species *Dickeya solani*, *Pectobacterium atrosepticum* and *Pectobacterium carotovorum* artificially inoculated on the surface of plant seeds from four species. Logarithmic reductions, as a key result of this work, were enclosed in the range of 1.61–4.95 in the case of *Cucumis sativus*, *Pisum sativum*, and *Vigna radiata*, while for the bacteria-inoculated *Zea mays* seeds, lower antibacterial properties of APPJ equaling 0.86–1.12 logs were noted. The herein applied exposure to APPJ did not reveal any statistically significant detrimental effects on the germination of plant seeds, seed coat integrity, or early plant growth. Even plant growth promotion by 20.96% was observed for the APPJ-exposed *Zea mays* seeds. By applying colorimetric assays and optical emission spectrometry (OES), we determined the oxidative potential in addition to identifying the reactive oxygen species (ROS) $^{\bullet}OH$, $^{\bullet}HO_2$, $^{\bullet}O_2^-$, O_3, and $^1O_2$ and the reactive nitrogen species (RNS) N, NO_2, and NO_3 responsible for the antibacterial properties of APPJ. In summary, universal antiphytopathogenic properties of the APPJ treatment reached due to proper optimization of the working conditions were revealed against three bacterial strains from the family *Pectobacteriaceae* inoculated on the seeds from diverse plant species. The data presented herein may contribute to future development of the plasma agriculture field and provide alternatives to pesticides or the prevention-based control methods towards plant pathogenic bacteria.

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1. Introduction

Agriculture is facing a tremendous challenge associated with the increase in the global population, which has already surpassed 8 billion people [1]. It is estimated that by 2050 the number of humans on Earth will reach 9.1 billion, and to feed such a population, food production must increase by 70% [1]. To ensure an adequate supply of high-quality nourishment, it is necessary to address contemporary issues related to crop losses associated with the presence of phytopathogens. Currently, up to 40% [2] of the crop yield is lost worldwide due to plant diseases, caused by various agrophages, e.g., nematodes, viruses, fungi, and bacteria. There are roughly 150 species of bacteria that are pathogenic to diverse plant species [3]. Owing to climate change, the ease of the spread of plant pathogenic bacteria and the risk of the occurrence of plant diseases will increase [4], resulting in a higher number of disease outbreaks and a notable decrease in the soil of a number of beneficial plant growth-promoting bacteria [4]. As an example, the recorded temperature changes and more frequently registered events of severe weather, such as hurricanes, may contribute to the potent expansion of the agrophages [4]. On the other hand, in addition to elongated vegetation periods, the elevated CO_2 concentrations may positively impact the amount of potato yields, contrarily to the quality of tubers, which, due to larger size, may possess higher concentrations of dry matter [5].

Nowadays, the commonly applied methods to control bacterial plant pathogens rely on sustainable cultivation, biocontrol, and the use of pesticides. Since the Green Revolution, pesticides have been responsible for a rise in global food productivity [6] and until today remain the main element of the integrated pest management strategy [7]. In 2021, global pesticide usage reached overall 3.53 tons, which corresponds with 2.26 kg per hectare [8]. Despite a significant role in limiting damage to crops, the application of these preparations is linked with notable drawbacks. A major hazard resulting from the usage of pesticides is the toxicity towards non-target organisms, even humans. In particular, these compounds pollute water and soil, exhibit negative impact on soil fertility, or inactivate plant beneficial microflora [9]. Furthermore, phytopathogens tend to increasingly gain resistance to chemicals [7]. In 2022, there was a proposal of Green Deal policy by the European Commission, which aims to cut down the use of chemical pesticides by up to 50% by 2030 [10]. For this reason, there is a high need for searching for alternative methods for limiting the spread of phytopathogens and, by these means, reducing losses in the food production sector.

Having this in mind, one could consider applying biopesticides; however, their current scale of usage is small, as these formulations represent only 2.5% of the whole pesticide market [11]. Such a small percentage of market share is associated with several drawbacks, being a narrow spectrum of activity, short shelf lives, weather sensitivity, delayed results, and the relatively high cost of the commercially available products [12]. Another approach for substituting pesticides might be utilization of antibiotics to limit bacteria-triggered damage in crops. It needs to be stressed that antibiotics are not permitted for use against plant pathogens in the European Union (EU) [13], though they are allowed in some countries in North and South America, as well as in Asia [14]. Currently, there are at least fifteen antibiotics used in agriculture, with five most commonly implemented, i.e., streptomycin, oxytetracycline, kasugamycin, oxolinic acid, and gentamicin [14]. Sadly, the use of antibiotics to control pests in agriculture poses a significant risk of development and spread of

drug resistance into human pathogens threatening modern medicine [7]. On this basis, we may conclude that antibiotics do not seem like a viable option in the plant protection field.

There are also plenty of naturally occurring resources to be considered for plant protection purposes [15], including phages, pathogen-resistant plant varieties, pathogen-antagonistic bacteria or fungi, natural active compounds, among others [16]. The efficacy of phage-based approaches has been demonstrated as early as in 1924 [17]. Views into the development of this method are focused on the proposal of better delivery systems and extending the shelf life of the formulations [15], as UV light easily inactivates phage particles [18]. The phage-based techniques also suffer from an extremely narrow spectrum of action, limited to even single strains. Passing to plant-resistant varieties, growers are not often convinced to use them due to higher costs of the planting material. In 2017, the research performed by Lamichhane et al. [19] contained a questionnaire completed by 63 biocontrol experts from over 20 countries, which marked that further studies in this field should be focused mostly on multiple interactions. Also, other features were noted, including application techniques, formulations, and instructions for growers, or just mentioning socioeconomic factors [19]. However, the most important aspect is that further optimization under laboratory conditions is needed before integration into agricultural systems. It is worth underlining that in the case of many plant pathogenic bacteria, prevention of disease symptoms development is the only available approach [20]. Therefore, accurate diagnosis of the disease-causing agent becomes crucial. There are plenty of options for the proposal of diagnostic techniques enabling early detection of the pathogens [21], which may be based on real-time PCR [22], multiplex PCR, loop-mediated isothermal amplification (LAMP) [23], fluorescent in situ hybridization (FISH), microarrays, sequencing (metagenomics, amplicon sequencing), or immunological techniques (enzyme-linked immunosorbent assay (ELISA), lateral flow immunoassay (LFIA) [24,25]).

On the other hand, there are physical methods that do not require registration procedures but can adversely affect plant growth or target beneficial microflora [20]. Physical methods, such as the utilization of hot water, warm dry air, or UV radiation [20] are also implemented for food preservation purposes and in greenhouse farming. Aiming to introduce a higher number of physical approaches to the plant protection sector, scientists have recently focused on the putative implementation of cold atmospheric pressure plasma-based (CAPP) technology into agriculture.

The term “plasma” was coined by Irving Langmuir in 1927. Overall, plasma is an ionized gas, which can be formed under the defined discharge atmosphere, including open-to-air, helium, argon, or nitrogen. Generally, plasma can be divided into two main types, such as hot plasma and cold plasma; however, most of the controlled plasma applications are based on the utilization of CAPPs [26]. During CAPP ignition, a variety of reactive oxygen (ROS) and nitrogen species (RNS), charged particles, free electrons, and electric fields are produced [27], making CAPP a universal tool for controlled applications in plasma medicine [28], environmental protection [29], food processing [30], and agriculture [31]. Considering that the controlled application of CAPP in agriculture is mainly associated with generation of ROS, RNS, and UV light of antimicrobial properties, this approach has been so far studied in terms of the inactivation of bacteria and fungi [32–38]. Though implementation of CAPP into agriculture may result in further benefits like plant growth promotion triggered by the plasma exposure [39–44], reduction in pesticide contamination in the environment [45], and effective remediation of soil and water [46,47].

Considering a high need for the proposal of novel methods to eradicate bacterial phytopathogens [48], we aimed at conducting for the first time multivariate optimization of the proposed CAPP-generating reaction-discharge system to achieve universal antibacterial properties of the plasma treatment without detrimental effects on the exposed

plant material. A unique plasma generator of controlled current voltage characteristics, producing CAPP in the form of an atmospheric pressure plasma jet (APPJ), was utilized for this purpose. The multivariate optimization of CAPP working conditions in order to find suitable CAPP parameters for the achievement of the highest antibacterial properties towards plant pathogenic bacteria without impairing plant growth relied on the design of experiments (DoE) along with the response surface methodology (RSM). Post defining optimal treatment conditions, the antibacterial properties of the applied APPJ system against phytopathogens from the genera *Dickeya* and *Pectobacterium* spp. were revealed on plant seeds of various sizes and porosity, in this case of *Cucumis sativus*, *Pisum sativum*, *Vigna radiata*, and *Zea mays*. Also, after CAPP treatment, the vital parameters associated with seed germination efficiency, seed coat integrity, and early plant growth were determined. Finally, optical emission spectrometry (OES) was employed for the identification of ROS and RNS responsible for the antibacterial properties of APPJ, while the oxidative potential of these species was estimated based on colorimetric assays. The herein-described research outcomes contribute to the development of the plasma agriculture field and prove the versatility of the CAPP-based approach after the proper optimization of the working parameters of the developed APPJ system.

2. Results and Discussion

2.1. Multivariate Optimization of the Working Parameters of the Portable APPJ System

Based on the analysis of variance (ANOVA) test, it was found that the model describing the effect of the working parameters x_1 (the He flow rate, in L min^{-1}), x_2 (the modulation frequency of the HV AC, in Hz), and x_3 (the duty cycle of this HV AC, in %) on the response of the system, i.e., the length of the sprouts (L, in mm) grown from the plasma-exposed mung bean seeds, was found statistically significant. The *p*-value for the model was 0.031 ($<<\alpha = 0.1$), while the *p*-values for the linear and square terms included in the model were 0.112 and 0.023, respectively, with the *p*-values of the separate terms being 0.057 (x_1), 0.372 (x_2), 0.037 (x_1^2), and 0.057 (x_2^2). On the other hand, the *p*-value for the lack-of-fit test was 0.725 ($>>\alpha = 0.1$); therefore, it could not be concluded that the model did not fit the data well; ergo, the model accurately fitted the data, and there was no need to transform the data and/or add the other terms. The determination coefficient (R^2), indicating the goodness of fit of the measured L values with the response surface regression equation, was fairly good, i.e., 62.5%. The equation of the developed model was as follows: $L = 309 - 116.4x_1 + 0.0443x_2 + 11.29x_1^2 - 0.000200x_2^2$.

Considering the above-presented response surface regression equation, it was evident that the working parameter x_3 related to the power of the AC supplied to the CAPP was statistically insignificant; hence, we decided to use the lowest possible value, i.e., 25%. The increase in the working parameter x_1 led to a decrease in the L (Figure 1), likely because of the lower production rate of the ROS and RNS under the conditions of the higher flow rates of He, e.g., because the supplied CAPP energy could be consumed to a greater extent to sustain the APPJ itself. In the case of the working parameter x_2, there was a maximum at 110 Hz, offering the highest L value. As such, for the following set of working parameters, x_1 = 4.5 L min^{-1}, x_2 = 110 Hz, and x_3 = 25%, the highest possible value of L = 16.2 mm was obtained according to the developed model. These working parameters were regarded as optimal as they did not impair plant growth and have been applied in all further experiments.

2.2. Antibacterial Properties of the Controlled APPJ Treatment

The efficiency of elimination of plant pathogenic bacteria from the genera *Dickeya* and *Pectobacterium* from the surface of plant seeds significantly varied depending on the

bacterial strain or the plant species (Table 1). The highest microbial inactivation rates, from 4.23 to 4.95 logs, were revealed for the *Vigna radiata* seeds. Also, notable APPJ-based bacterial eradication rates were achieved from the surface of artificially inoculated *Cucumis sativus* (1.61–3.28 log reductions) and *Pisum sativum* (1.61–4.79 log reductions) seeds.

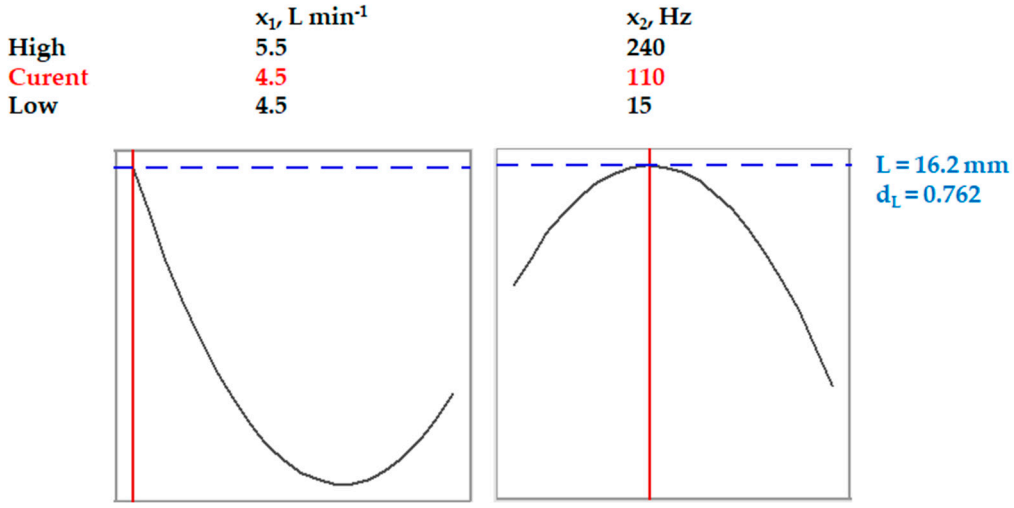

Figure 1. Constituents of the implemented model affecting the response of the system. The effect of the working parameters x_1 (the He flow rate, in L min^{-1}) and x_2 (the modulation frequency of the HV AC, in Hz) on the measured response L (the length of sprouts, in mm) of the system.

Table 1. The percentage (and logarithmic) reductions in the number of viable cells of soft rot *Pectobacteriaceae* artificially inoculated on the surface of various plant seeds. The percentage reductions in the number of colony-forming units (CFU) per mL of the Ringer buffer were shown. A 0.5 McFarland (McF ≈ 1–2 × 10^8 CFU mL^{-1}) density of the bacterial suspension was implemented for seed inoculation. Statistically significant differences between treatment groups for a single plant species are indicated by diverse letters as determined by Kruskal–Wallis test followed by applying the post hoc Fisher's least significant difference criterion ($p < 0.05$). The experiment was repeated three times with three technical replicates.

Bacterial Strain	*Cucumis sativus*	*Pisum sativum*	*Vigna radiata*	*Zea mays*
Dickeya solani	99.6451% ± 0.6045% (2.45) ab	99.9984% ± 0.0024% (4.79) a	99.9989% ± 0.0019% (4.95) a	88.2043% ± 9.5591% (0.93) d
Pectobacterium atrospeticum	97.539% ± 7.3272% (1.61) a	98.2651% ± 2.355% (1.76) bc	99.9942% ± 0.0048% (4.23) a	92.4406% ± 10.1985% (1.12) c
Pectobacterium carotovorum	99.9477% ± 0.0722% (3.28) a	97.5813% ± 2.25% (1.61) bc	99.9977% ± 0.0019% (4.64) a	86.2014% ± 16.002% (0.86) d

The lowest percentage reduction rates, varying from 0.86 to 1.12 logs, were noted for plant pathogenic bacteria inoculated on the surface of *Zea mays* seeds. The size, shape, and irregular surface of these seeds presumably impeded the efficacy of the APPJ treatment. It is also worth noticing that the *Zea mays* seeds were the biggest ones examined in this study. In another piece of research by Zahoranová et al., 100% elimination of the naturally occurring bacteria from the maize seeds after 60 s APPJ exposition and similarly total inactivation of fungi after 180 s treatment have been achieved with the use of Diffuse Coplanar Surface Barrier Discharge (DCSBD) [43]. Though, it needs to be stressed that significantly lower initial microbial counts were determined in that research (5 × 10^1 CFU per gram of seeds concerning bacteria and 3.2 × 10^3 CFU/g of seeds regarding filamentous fungi) in contrast to the current one. Also, as stated before, Zahoranová et al. eliminated natural microflora in the case of fungi from the genera *Aspergillus*, *Fusarium*, *Penicillium*, *Cladosporium*, and

Verticilium, while the exact bacterial species have not been identified in that research. In addition, artificial inoculation-based experiments with several fungal species have been conducted there. Namely, a 2 min of DCSBD treatment led to 100% inactivation of *Fusarium culmorum* cells (initial dose—6.0×10^3 CFU/g), the 3.15 ± 1.75 log reduction in the number of viable cells of *Aspergillus flavus*, and the 2.18 ± 1.32 log reduction in terms of *Aspergillus alternata*. Not only did the CAPP-generating systems differ between the current research and the one of Zahoranová et al., but we also focused on bacterial pathogens in contrast to fungal pathogens. Putatively, a diffused coplanar surface APPJ with a 20 cm × 8 cm highly active plasma area used by Zahoranová et al. could effectively affect microbial cells even from more difficult-to-reach areas of the maize seeds. Aiming for large-scale applications of the herein-reported reaction discharge system, we foresee that together with scaling up the device, we would be able to address the issues associated with areas that are difficult to reach by the plasma jet, alike to the improvements we introduced into a reaction-discharge system dedicated to the inactivation of antibiotics [49]. In this way, it would be possible to demonstrate all the potential of the APPJ without drawbacks linked with a limited exposition area. Anyway, high inactivation rates for three plant pathogenic bacteria have been achieved here from at least three plant seed types, although enlarging the panel of the tested microbial and plant species would address the limitations of the current study.

Referring to the so-far proposed mechanisms behind the phenomenon of CAPP-mediated microbial inactivation, there are often listed cell etching due to reactive species, the volatilization of intrinsic compounds from the cell, and the destruction of genetic material [50]. Furthermore, interactions between ROS, RNS, and macromolecules inside or on the surface of the microbial cell lead to denaturation of proteins and leakage of the cell contents [51]. Though, it has been suggested that the mechanism of bacterial inactivation may differ depending on the structure of the microbial cell wall. For instance, Han et al. pointed out that the APPJ-treated Gram-negative bacteria were eliminated mainly due to the CAPP-induced destruction of the cell envelope resulting in cellular leakage. On the contrary, Gram-positive bacteria underwent inactivation mostly by the CAPP-triggered intracellular damage [52]. Also in our previous study, we observed outflow of the cytoplasm and condensation of the components of the protoplast in the APPJ plasma-exposed cells of Gram-negative plant pathogenic *D. solani*, visualized under the transmission electron microscopy [35].

2.3. Effect of the APPJ Exposure on the Seed Coat Integrity

Due to damage affecting the seed coat, an increase in permeability can occur, which may be measured with the conductometer by determination of a degree of leakage of the electrolytes to the external medium [53]. Thus, we undertook this methodology to state whether the APPJ exposure leads to losses in the seed coat integrity, and the conductivity measurements have been performed shortly after CAPP treatment (0 h), 1 h, 2 h, and 24 h post-subjection to the plasma source. The collected results pointed to no statistically significant differences between the seed coat integrity of the seeds subjected to APPJ in contrast to the corresponding controls 24 h after the treatment (Figure 2). The study conducted by Sayahi et al. on the effect of CAPP exposure on soybean seeds indicated that solely after a 180 s or longer APPJ exposure, the conductivity of the solution in which the seeds were submerged increased [42]. In another study, mung bean seeds treated by CAPP generated in an air-based DBD discharge system showed significant disturbances of the seed coat integrity even after a short 20 s treatment time [54]. In general, most of the literature references report higher conductivity values for the APPJ-treated groups in juxtaposition to the controls. Contrarily, in the current research, in the case of almost all

plant species and the examined post-CAPP exposure periods, there were no statistically significant differences between the APPJ-treated seeds and untreated controls in terms of the seed coat integrity. There was only one notable exception of the mung bean seeds, which shortly after CAPP subjection and 1 h post-treatment showed even lower degrees of electrolyte leakage than the corresponding untreated control (Figure 2). The results obtained herein confirm that there is no harmful effect of the application of APPJ on the structure of the seed coat. This parameter is important for the determination of the quality of the planting material. Furthermore, it is associated with the efficacy of the seed germination process, and distortion of the seed coat integrity may lead to the increased susceptibility to penetration by various phytopathogens [55].

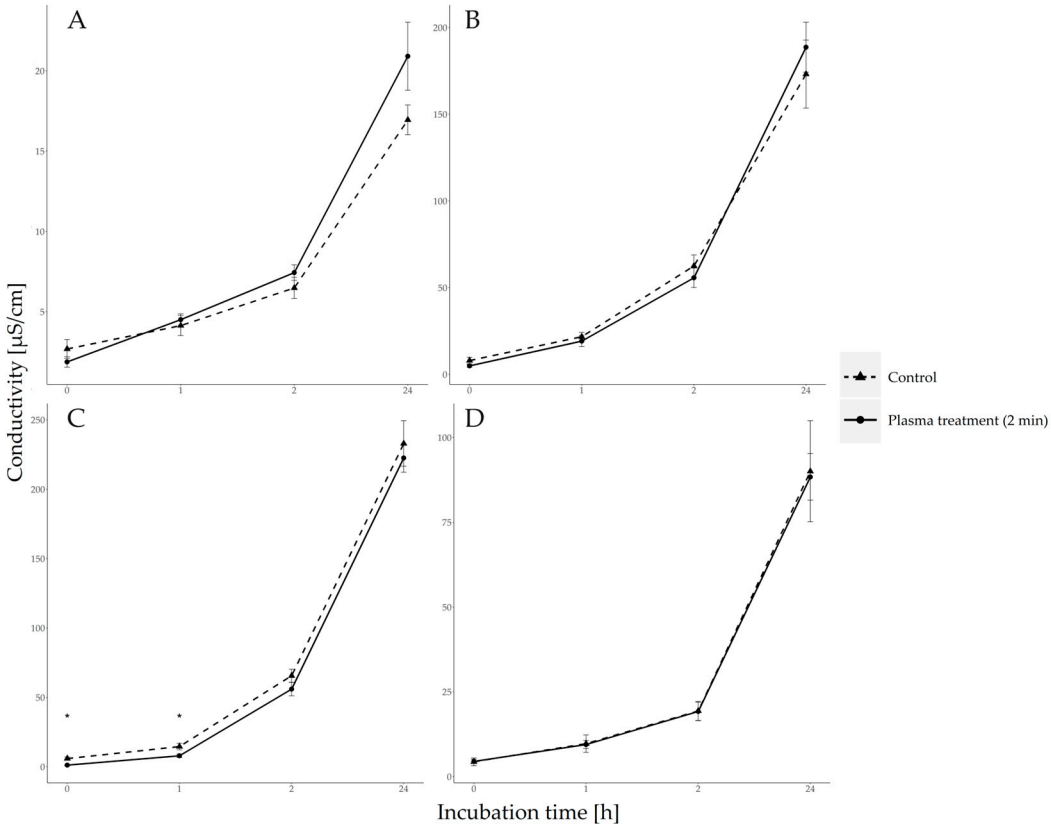

Figure 2. Assessment of the seed coat integrity by the conductivity measurements of solute leakage from the APPJ-exposed seeds of: (**A**) *Cucumis sativus*; (**B**) *Pisum sativum*; (**C**) *Vigna radiata*; (**D**) *Zea mays* in relation to controls that were not treated with CAPP. The seeds were exposed to the APPJ for 2 min, and after the treatment, they were immersed in deionized water. Each point (plasma-treated seeds) or triangle (control) corresponds to the mean conductivity ± standard errors. The asterisk marks a statistically significant difference between the untreated control and the treated sample (Welch's test at $p < 0.05$). The examined incubation periods involved 0, 1, 2, and 24 h post CAPP exposure.

2.4. Impact of the Optimized APPJ Treatment on Seed Germination and Early Plant Growth

The percentage of the germinated seeds was determined in 24 h intervals for a period of 7 days. In every APPJ-treated group versus the corresponding control for each of the four examined plant species, no statistically significant differences were noted (Figure 3). In addition to each of the investigated 24 h intervals, the latter observation was valid for the final percentage of the germinated seeds (Table S1). According to the literature, CAPP application can be beneficial for early plant growth [40], mostly owing to the generated active species, i.e., RONS that can easily penetrate through the seed coat and directly impact the plant cells. Also, due to damage and ablation on the surface of the seeds caused by the CAPP source, the transmission of O_2 and moisture tends to be enhanced, which

finds reflection in the recorded germination rates. Additionally, ROS generated by CAPP influence the GA/ABA balance, which is crucial during the onset of seed germination [56]. The ABA signaling pathway is activated in dormant seeds, while GA-dependent signaling exhibits a tendency to be reduced. Bahin et al. reported that H_2O_2 treatment changes this pattern, favoring GA signaling and the promotion of the seed germination process [57].

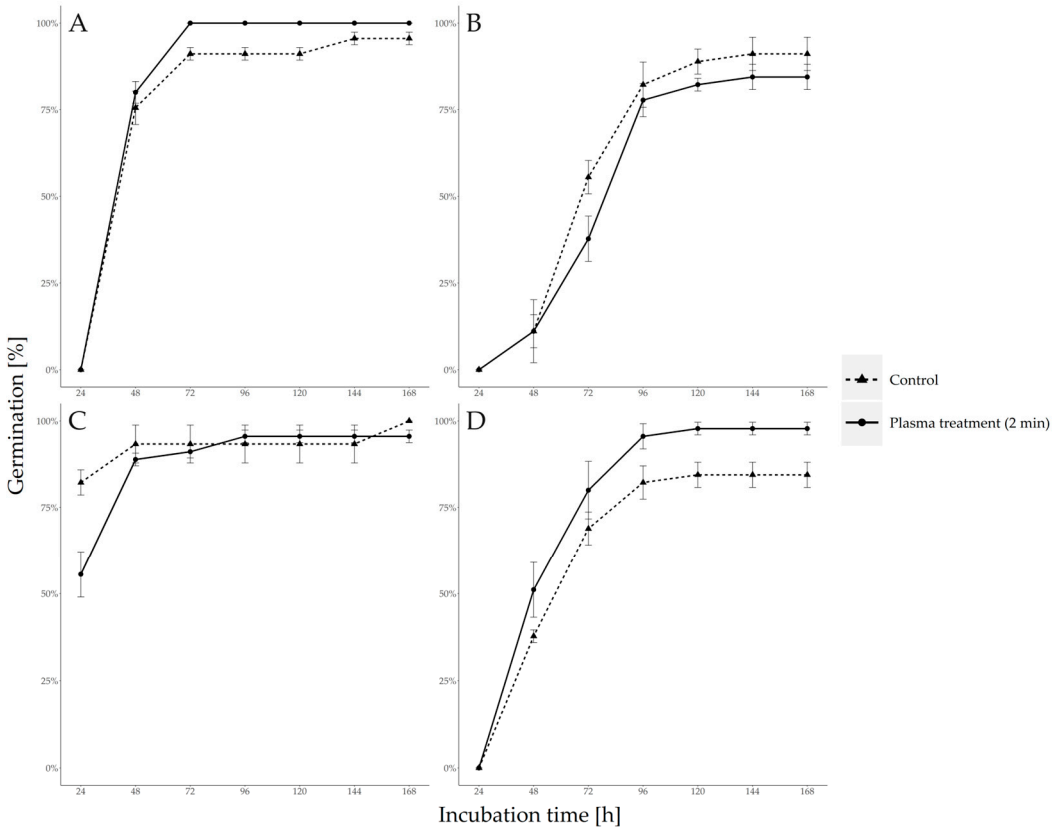

Figure 3. The effect of the APPJ treatment on the germination rate of: (**A**) *Cucumis sativus*; (**B**) *Pisum sativum*; (**C**) *Vigna radiata*; (**D**) *Zea mays*. The exposure of the seeds to the APPJ lasted 2 min. Each point (plasma-treated seeds) or triangle (control) corresponds to the mean cumulative percentage of the germinated seeds at a stated time period with ± standard errors presented on the whiskers. No statistically significant differences between the untreated control and the treated samples have been observed in the case of seeds from all the investigated plant species (Welch's test at $p < 0.05$).

It is also worth acknowledging the noted reduced germination time spread for cucumber and mung bean seeds, which is the difference between the time that passed from the last germination to the first germination event (Table S1). According to Melville et al. [58], the recorded higher germination index refers to a more rapid process of seed germination. The latter parameter was revealed to be higher for the APPJ-treated seeds of *Cucumis sativus* and *Zea mays* in comparison to the untreated controls, which corresponds to a slightly higher final seed germination percentage in terms of these two plant species. Interestingly, Holubová et al. observed that 2 min of the application of nitrogen CAPP to maize seeds resulted in the complete inhibition of the germination of maize seeds. Also, the implementation of ambient air or oxygen as discharge gases significantly decreased the achieved germination rate [41]. This observation has been linked with the production of a high dosage of ultraviolet (UV) radiation during CAPP generation, which had a detrimental effect on the seed germination process. Also, in that work, it was confirmed that nitrogen plasma is responsible for the most intensive UV radiation. Nevertheless, shorter CAPP treatment periods (30 s, 60 s) led to maize seed germination rates that were comparable to the untreated control samples [41].

Despite focusing on seed germination, we also examined early plant growth, which was determined based on the length of the sprouts formed from the APPJ-exposed seeds in relation to the corresponding controls. In the case of *Cucumis sativus*, *Pisum sativum*, and *Vigna radiata* seedlings, no statistically significant differences were noted between the mean length of the APPJ-treated groups and the corresponding controls; therefore, no impact of the CAPP exposure on early plant growth was concluded regarding the above-listed three plant species (Figure 4). The sole noted statistically significant positive effect in this experiment concerned promotion of the early growth of *Zea mays* sprouts by 20.96% due to APPJ treatment (Figure 4).

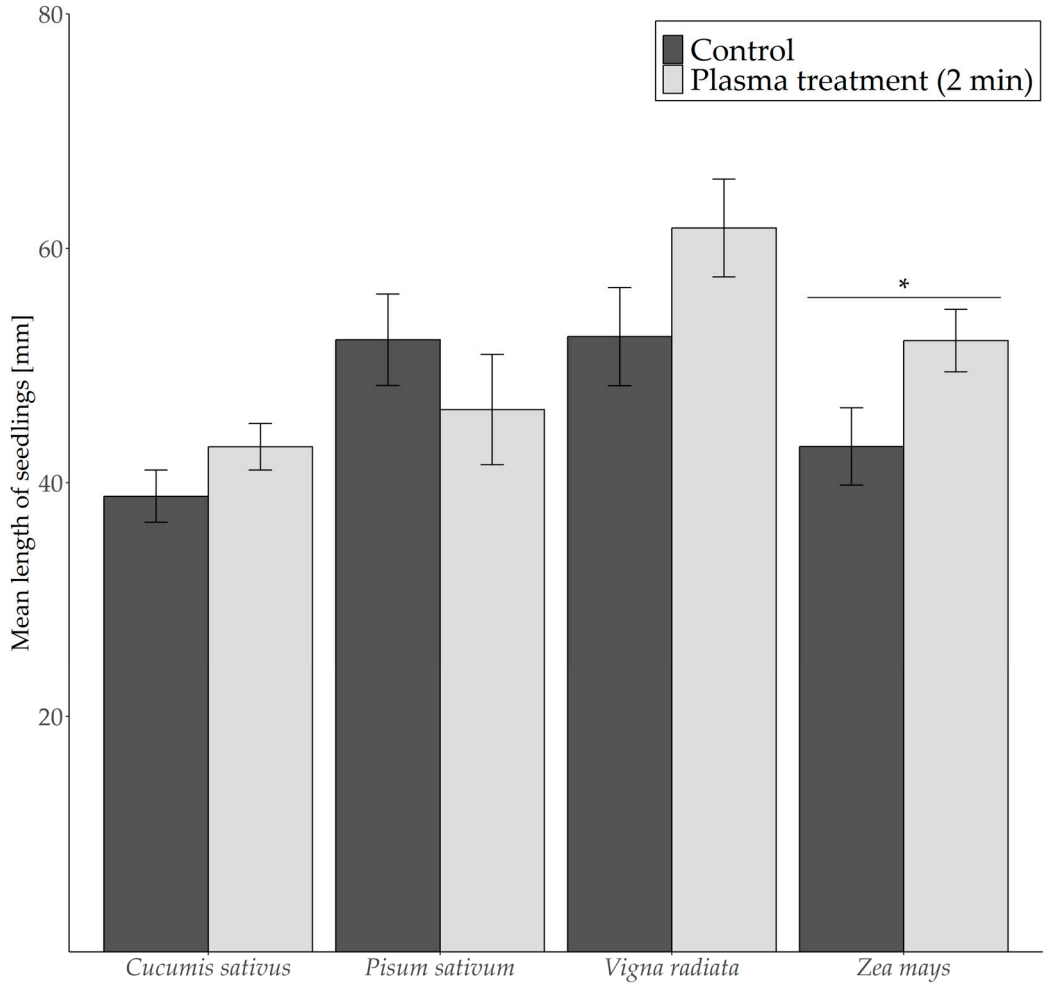

Figure 4. The effect of the CAPP treatment on the mean seedling length of the examined plant species. The exposure of the seeds to the APPJ lasted for 2 min. Bars correspond to means ± standard errors. The asterisk marks a statistically significant difference between the untreated control and the treated sample (Welch's test at $p < 0.05$).

In the current research, the effective eradication of phytopathogens from the seed surface, in addition to the non-harmful effect of the application of APPJ on the seed germination and the development of the examined plant species, confirmed the potential of CAPP-based technology as a tool for agricultural purposes. As already mentioned, there is a constant need for eco-friendly, innovative solutions to improve the quality of the crops and quantity of the generated yield. In the future, CAPP, due to its pro-ecological nature, ease of operation, and cost-effectiveness, may be widely used by seed producers or growers, but before a comprehensive assessment of the benefits and risks of standalone processes

involving an APPJ unit or integration of this methodology into the processing chain is highly needed [31].

2.5. The Reactive Nitrogen and Oxygen Species Produced During APPJ Exposure Conducted Under the Optimal Working Conditions

The emission spectrum of the APPJ system is presented in Figure 5. As can be observed from Figure 5A, in the UV region (200–400 nm), the OH (A-X) and N_2 (C-B) emission bands were excited. The main bands of OH (A-X) were noted at 282.9 nm (1-0 transition) and at 308.9 nm (0-0 transition). The UV spectrum of APPJ was also dominated by numerous N_2 (C-B) bands with the band head at 315.9 nm (1-0), 337.1 nm (0-0), 353.7 nm (1-2), 357.7 nm (1-2), 375.5 nm (1-3) and 380.5 nm (0-2). Additionally, a strong N_2^+ (B-X) band at 391.4 nm (0-0) was noticed.

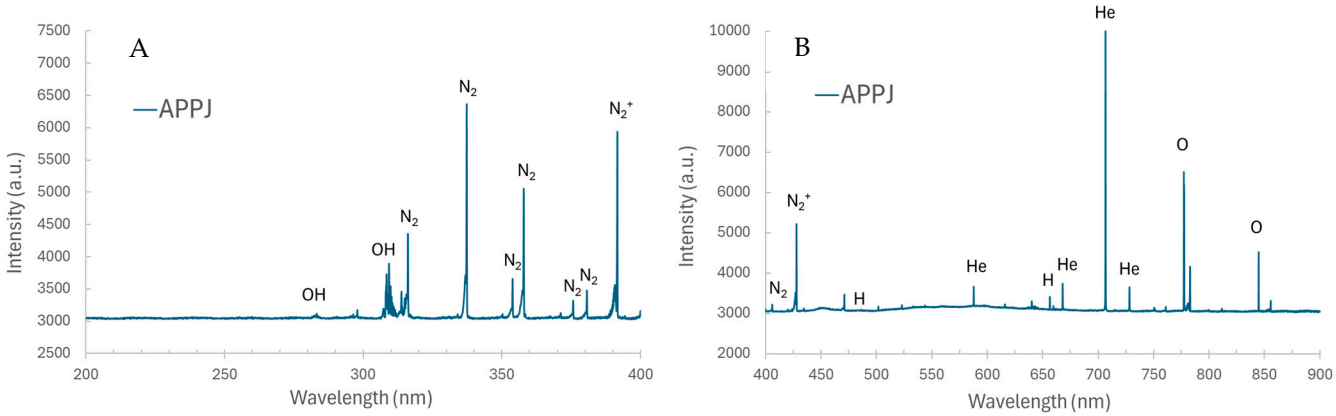

Figure 5. The emission spectrum of the APPJ system in the (**A**) 200–400 nm and (**B**) 400–900 nm regions.

In the UV-NIR region (400–900 nm) (Figure 5B), the He atomic lines (He I) were noted at 587.5 nm, 667.8 nm, 706.5 nm, and 728.1 nm. Additionally, the atomic lines of H at 486.1 nm and 656.2 nm and the atomic lines of O (O I) at 777.2 nm, 772.4, and 844.6 nm were also revealed. The above-described outcomes pointed to the formation of the following ROS and RNS, i.e., OH, N_2, N_2^+, O, as well as H, and He, by the examined APPJ system.

Subsequently, colorimetric assays based either on degradation of methylene blue (MB) or 4-nitrophenol (4-NP) were conducted to obtain a better insight into specific ROS produced by the implemented APPJ source, working under the defined optimal conditions. It should be stressed that the degradation process of MB is mainly driven by ROS such as O_3, $^1O_2$, $^\bullet$OH, $^\bullet HO_2$, and $^\bullet O_2^-$ produced during APPJ operation [59,60]. As can be seen in Figure 6, the degradation of MB reached 42% ± 3.62% within 2 min of the APPJ treatment. It suggests significant CAPP-mediated production of radicals, along with O_3 and O, as revealed by the MB degradation process. According to the literature [61], the oxidative potential of ROS responsible for MB degradation should be sufficient for the herein observed bactericidal effect.

In order to further study the types of ROS produced during APPJ ignition, the assay based on degradation of 4-nitrophenol (4-NP) was included, aiming for assessment of the contribution of hydroxyl radicals into this process (Figure 6) [62,63]. Despite the significant resistance of this organic pollutant, 29.55% ± 3.97% of 4-NP was degraded following 2 min of APPJ treatment. It should be emphasized that from the broad spectrum of ROS representatives generated by the examined APPJ system, the participation of the formed $^\bullet$OH remains distinct. On the other hand, during the multivariate optimization of APPJ operational conditions, the length of the sprouts was selected as a response of the system. It can be summed up that the optimized APPJ discharge parameters might favor the enhanced generation of reactive nitrogen species that greatly support the growth of sprouts [64,65].

Despite the well-recognized oxidative potential of the optimized APPJ, the above-described optical spectroscopy studies provided a broader view of the chemical composition of the reactive individuals formed by the implemented CAPP discharge.

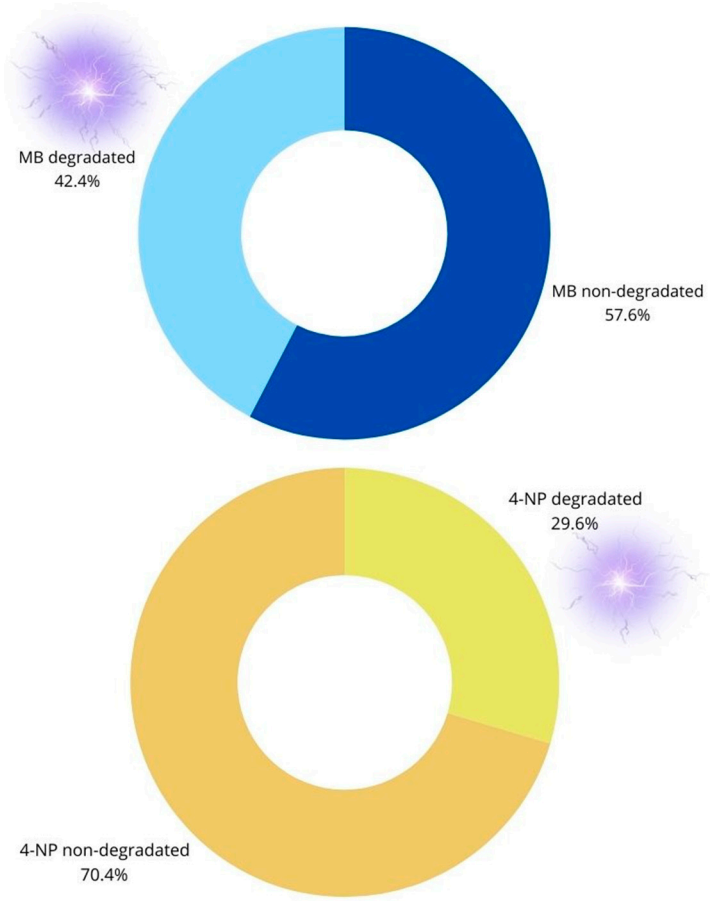

Figure 6. The oxidative potential of APPJ treatment as determined by the degradation of methylene blue and 4-nitrophenol from the solutions within a 2 min treatment time.

3. Materials and Methods

3.1. The Implemented Portable APPJ System

For the ignition of CAPP, APPJ was used as a plasma source (Figure 7). The implemented CAPP-based system was developed by our research group and described in detail in our previous study [35]. Briefly, the APPJ was generated using a plasma generator (Dora Electronics Equipment, Wilczyce, Poland). Helium was used as a discharge gas (4.0, Linde Poland, Krakow, Poland). For the achievement of the optimal operating conditions, the selected parameters, such as the flow rate of the helium (4.5–5.0 L min^{-1}), the modulation frequency of the HV AC (15–240 Hz), and the duty cycle (25–75%), were chosen as crucial parameters (see Section 3.2 for more details on the optimization of the working conditions). The exposure of the plant seeds to the APPJ lasted 2 min during all experiments, and the plasma stream (a gap between the end of a quartz tube of the system through which the plasma tip was extended and the treated material) equaled 2 cm.

The optimal working parameters at which APPJ was sustained and operated stably were as follows: a modulation frequency of 170 Hz, a duty cycle of 25%, and a He flow rate of 4.5 L min^{-1}.

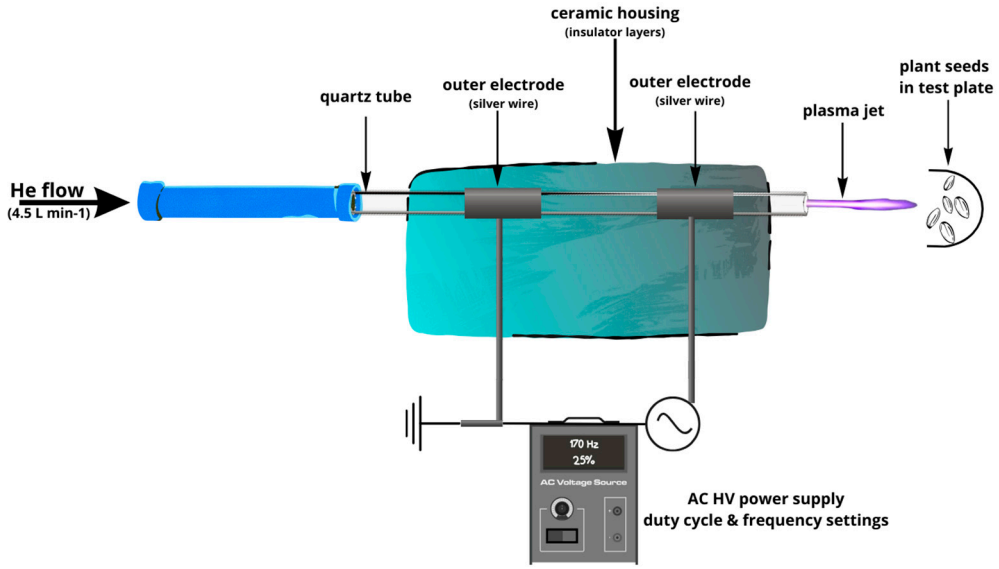

Figure 7. Scheme of the implemented CAPP-based system.

3.2. Multivariate Optimization of the APPJ Working Parameters

In order to achieve the antibacterial properties of the APPJ exposure without detrimental effects on the plant material, the selected working parameters of the applied CAPP system were optimized. The following working parameters were considered: the He flow rate (x_1, in L min^{-1}), the modulation frequency of the HV AC (x_2, in Hz), and the duty cycle of this AC (x_3, in %). The optimization of these parameters was carried out by applying the Box–Behnken response surface design. In this case, the working parameters x_1, x_2, and x_3 were set at three levels, i.e., "−1" (low), "0" (middle), and "+1" (high), and randomly combined for the given experimental treatment. The above-mentioned boundaries "−1", "0", and "+1" were selected based on the stable operation of the APPJ, and they were as follows: 4.5, 5.0, and 5.5 L min^{-1} for x_1; 15, 128, and 240 Hz for x_2; and 25, 50, and 75% for x_3. During the response surface design optimization with the Box–Behnken design, fifteen experimental treatments were planned and executed according to the run order given in Table 2. Three center points, i.e., the experimental treatments carried out at the working parameters set to the middle values (nos. 1, 8, and 9), were also included in them and allowed to assess the reproducibility of the measured response. The response of the system was the mean length of the sprouts (L, in mm) formed from five mung bean seeds per treatment group that were exposed to the APPJ at the given working parameters. The L, changing from 1 to 5 mm, was measured for all five mung bean sprouts per group and averaged. All the experimental treatments were performed in one block. The reproducibility of the experiments within the Box–Behnken response surface design optimization, as considered the relative standard deviation of the L values measured for the center points (11.3 mm ± 2.7 mm), was fair and equaled to 23%. The values of the response L (in mm) were approximated with the following full quadratic function, i.e., $a_0 + a_1x_1 + a_2x_2 + a_3x_3 + a_{11}x_1^2 + a_{22}x_2^2 + a_{33}x_3^2 + a_{12}x_1x_2 + a_{13}x_1x_3 + a_{23}x_2x_3$, including the linear, square, and two-coefficient terms. The statistically significant terms and the values of the intercept (a_0) and the regression coefficients (a_1, a_2, a_3, a_{11}, a_{22}, a_{33}, a_{12}, a_{13}, a_{23}) were established by using the background-elimination-of-terms procedure (at $\alpha = 0.1$) and the ANOVA of the residuals between the measured values at given sets of the working parameters and the values modeled with the developed response surface regression equation was determined for these working parameters. All analyses and visualizations of the data related to the multivariate optimization were carried out using the statistical software Minitab 17.1.0 (Minitab Inc., USA).

Table 2. The run order of the experimental treatments carried out within the Box–Behnken response surface design multivariate optimization of the He-DBD plasma jet.

Run Order	x_1	x_2	x_3
1 [$]	5.0 (0)	128 (0)	50 (0)
2	5.0 (0)	15 (−1)	25 (−1)
3	4.5 (−1)	128 (0)	75 (+1)
4	5.0 (0)	240 (+1)	75 (+1)
5	5.0 (0)	15 (−1)	75 (+1)
6	4.5 (−1)	240 (+1)	50 (0)
7	4.5 (−1)	128 (0)	25 (−1)
8 [$]	5.0 (0)	128 (0)	50 (0)
9 [$]	5.0 (0)	128 (0)	50 (0)
10	5.5 (+1)	15 (−1)	50 (0)
11	5.5 (+1)	128 (0)	25 (−1)
12	4.5 (−1)	15 (−1)	50 (0)
13	5.5 (+1)	128 (0)	75 (+1)
14	5.5 (+1)	240 (+1)	50 (0)
15	5.0 (0)	240 (+1)	50 (0)

[$] The center points. x_1—The He flow rate (in L min^{-1}). x_2—The modulation frequency of the HV AC (in Hz). x_3—The duty cycle of the HV AC (in %).

3.3. Biological Material

3.3.1. Bacterial Suspensions

Three strains of phytopathogenic bacteria, i.e., *Dickeya solani* IFB0099, *Pectobacterium atrospeticum* IFB5103, and *Pectobacterium carotovorum* IFB5118, were utilized in this studies (Table 3). All strains belong to the collection of the Intercollegiate Faculty of Biotechnology University of Gdansk and Medical University of Gdansk (Gdansk, Poland) and were stored at −80 °C in 40% glycerol.

Table 3. Characterization of the utilized strains of phytopathogenic bacteria.

Bacterial Species	Strain Nos.	Plant of Origin (Organ)	Diseases	Country of Isolation	Year of Isolation	Reference
Dickeya solani	IFB0099 (IPO2276; LMG28824)	Potato (Tuber)	Blackleg and soft rot	Poland	2005	[66]
Pectobacterium atrospeticum	IFB5103 (SCRI1086)	Potato (Tuber)	Blackleg and soft rot	Canada	1985	SCRI collection
Pectobacterium carotovorum	IFB5118 (SCRI1136)	Potato (Tuber)	Soft rot	USA	NA	SCRI collection

NA—not available.

Bacterial biomass was obtained from frozen stock and streaked on Trypticase soy agar (TSA) medium. After 24 h of incubation at 28 °C, a single bacterial colony was taken to inoculate 5.0 mL of Trypticase soy broth (TSB). The 0.5 McF bacterial suspensions were prepared in 0.85% NaCl as follows. Post 24 h incubation at 28 °C with 150 rpm shaking, the overnight bacterial culture was centrifuged (10 min, 6500 rpm). The obtained pellet was washed twice with sterile 0.85% NaCl. After that, the optical density of the bacterial suspension was set at 0.5 McF (≈1–2 × 10^8 CFU mL^{-1}) by using a densitometer DEN-1B (Biosan, Riga, Latvia). The 0.5 McF suspensions of plant pathogenic bacteria were used to evaluate the antibacterial activity of the APPJ operated under the optimal working parameters.

3.3.2. Plant Material

The efficacy of bacterial elimination from the surface of plant seeds, in addition to the impact of the APPJ exposure on plant material (seed germination potential, seed coat integrity, and early plant growth), was examined for seeds of 4 different plant species: *Cucumis sativus* (cucumber), *Pisum sativum* (pea), *Vigna radiata* (mung bean), and *Zea mays* (maize).

Before the seeds were exposed to the APPJ, they were disinfected by immersion in a 70% ethanol solution for 30 s. After 1 min of washing in sterile distilled water, the seeds were drowned in 3% H_2O_2 for 10 min. The disinfected seeds were washed four times in distilled water to remove disinfectant residues. Seeds disinfected in that manner were used in all the conducted assays as described below.

3.4. Inactivation of the Cells of Plant Pathogenic Bacteria from the Surface of Plant Seeds by the Applied APPJ Treatment

The antimicrobial activity of the applied APPJ system against plant pathogenic bacteria was examined on the seeds of four different plant species. In this experiment, five disinfected seeds from one plant species per replicate and inoculated with one bacterial strain by placing them in a single well of a 12-well microplate and flooding with the 0.5 McF suspension of microbial cells for 30 s with shaking. After that, the seeds were transferred with sterile tweezers to a new 12-well microplate and treated with the APPJ, sustained and operated under the optimal working parameters, for 2 min from a distance of 2 cm. During the exposure, the 12-well microplate was gently shaken to ensure even access of the seed surfaces to the plasma stream. Disinfected as described above and artificially inoculated seeds, which have not been subjected to the APPJ treatment, were included as the control samples in this procedure. All the samples, the treated ones and the controls, were then immersed separately in ¼ Ringer solution (Oxoid, Hampshire, UK) and shaken at 1200 rpm. The Ringer solution with the transferred bacterial cells was then serially diluted up to 10^{-5}. A total of 100 µL of every dilution was spread on a TSA medium and incubated for 48 h at 28 °C. After incubation, the grown bacterial colonies were counted and recalculated to CFUs per mL of the Ringer's buffer. Subsequently, the percentage and logarithmic reductions in the number of viable bacterial cells were counted for the samples treated by the APPJ in contrast to the untreated controls. The experiment was repeated 3 times with 3 technical replicates for each plant species and every bacterial strain.

3.5. Influence of the APPJ Treatment on Seed Germination Efficacy

In the case of the seed germination experiment, 5 disinfected seeds of each plant species were transferred with sterile tweezers into a single well of the 12-well microplate. There, each 5-seed set was exposed to APPJ treatment, which operated under the optimal working parameters, for 2 min and at a distance of 2 cm. The 12-well microplate was gently shaken to ensure even access of the plasma stream to the surface of the seeds. The disinfected and artificially inoculated seeds that were not treated by the APPJ were included as control samples. Afterwards, each set of five seeds treated by the APPJ or the untreated control seeds was put on a filter paper (type MN 640 W; Macherey-Nagel, Duren, Germany) on a Petri plate (90 mm diameter) and watered with 5 mL of sterile distilled H_2O. The lid-covered plates with the plant seeds were incubated in a plant growth chamber at 16 h light (120 µmol m^{-2} s^{-1}, 20 °C) and 8 h darkness (18 °C) conditions. The incubation lasted 168 h, during which the number of the germinated seeds was recorded daily. The collected results were analyzed in terms of the single-value germination indices for describing the time course of the germination process. The experiment was repeated three times with three technical replicates.

3.6. Effect of APPJ on Integrity of the Seed Coat

In this assay, 5 disinfected seeds (as described above) were transferred into a single well of the 12-well microplate. There, each 5-seed set was exposed to the APPJ treatment, which operated under the optimal working parameters, for 2 min from a distance of 2 cm with provision of gentle shaking. Then, 5 seeds were immersed in 30 mL of sterile distilled H_2O in 50 mL-volume Corning® falcon tube (Corning, NY, USA). The conductivity measurements were performed with an HI763100 probe connected to an HI2020-01 edge™ laboratory meter (Hanna Instruments, Olsztyn, Poland). The conductivity of the water with immersed seeds was measured shortly after CAPP exposure (0 h), post 1 h, 2 h, and 24 h periods. The experiment was repeated three times with three technical replicates.

3.7. The Impact of the APPJ Treatment on the Early Plant Growth

The seeds belonging to four plant species were disinfected, exposed to CAPP, and further handled as described in Section 3.5. After the 168 h incubation period in the plant growth chamber (16 h of light at 120 μmol m^{-2} s^{-1} at 20 °C; 8 h of darkness at 18 °C) the lengths of the sprouts grown from the APPJ-treated seeds in contrast to the controls were measured. The experiment was repeated three times with three technical replicates.

3.8. Identification of ROS and RNS Generated in the Applied APPJ Source

Considering the close interactions between APPJ and the seeds artificially inoculated with plant pathogenic bacteria, the antimicrobial effect should be driven directly by reactive species of a short lifespan and showing a high oxidative capability.

In order to identify the ROS and RNS produced during APPJ operation, OES was used. This instrumental technique allowed us to characterize the gaseous phase of APPJ. For OES measurements, a high-resolution Shamrock SR500 spectrograph (ANDOR, Abingdon, UK) was implemented. The OES spectrograph was equipped with a CCD camera (Newton DU-920P-OE, Andor, UK) cooled to −65 °C, and two gratings: 1800 and 1200 grooves per mm, for the spectral regions of 200–400 nm and 400–900 nm, respectively. The CCD detector operated in FVB (full vertical binning) mode with an integration time of 0.1 s. For processing and acquisition of the APPJ spectra, the Solis S software v 4.32(ANDOR, UK) was used. The emission from the APPJ tip was imaged onto the spectrograph's entrance slit (10 μm) using a UV achromatic lens (f = 60). The APPJ spectra in the 400–900 nm range were measured with an additional cut-off (<380 nm) WG5 filter (Zeiss, Jena, Germany).

To determine which specific ROS and RNS are mainly generated for the studied APPJ system, experiments involving the degradation of MB and 4-NP were performed. In terms of the first assay, 0.01 g L^{-1} MB solution (Avantor Performance Materials, Gliwice, Poland) was prepared according to good laboratory practice. Then, the resultant solution was transferred to the 24-well test microplate. The degradation experiments were performed in three repetitions under optimal APPJ conditions, maintaining a distance of 2 cm from the liquid surface, while the treatment time was 2 min. After APPJ treatment, the MB solution was transferred into quartz cuvette and the absorbance value was measured at 664 nm using a Specord 210 Plus UV/Vis spectrophotometer (Analytik, Jena, Germany). The effectiveness of APPJ application was presented as the degradation percentage referring to the absorbance values of the not-treated MB solution. The experiment was conducted with three technical replicates.

In the second experiment, a 1 mM solution of 4-NP (Avantor Performance Materials, Poland) was prepared. A total of 2.00 mL of the obtained solution was transferred to the 24-well test microplate. The degradation process involving the APPJ source was evaluated in the same manner as for the MB degradation. The post-treatment 4-NP solution was transferred into a quartz cuvette, and a spectral scan from 200 to 600 nm with a

scan speed of 50 nm/s and step 0.5 nm was acquired using a Specord 210 Plus UV/Vis spectrophotometer. The maximum absorbance determined from the spectral scan at 318 nm was used to determine the degradation efficiency of 4-NP, and the results were presented as degradation percentage referenced to the absorbance values for the not-treated 4-NP solution. In all experiments, deionized water was used throughout. The experiment was conducted with three technical replicates.

3.9. Data Visualization and Statistical Analysis

The collected data were visualized and statistically analyzed using R v 4.2.2 [67]. For the statistical analysis, the *agricolae* and *onewaytests* packages were utilized. To evaluate the equality of variances and data normality, Levene's and Shapiro–Wilk's tests were conducted, respectively. Welch's two-sided *t*-test was applied to check the statistical significance of the differences between the samples treated by the APPJ and the control samples. In terms of evaluating the susceptibility of different bacterial species to the APPJ treatment, as the ANOVA requirements were not met, the Kruskal–Wallis test was used and followed by applying the post hoc Fisher's least significant difference criterion. $p < 0.05$ was utilized for all calculations. The analysis of single-value germination indices was carried out using the *germinationmetrics* package [67].

4. Conclusions

Due to the conducted multivariate optimization of the APPJ operating conditions, universal antibacterial properties against plant pathogens *D. solani*, *P. atrosepticum* and *P. carotovorum*, varying from 1.61 to 4.95 logs, were achieved from the surface of artificially inoculated plant seeds from three species, i.e., *Cucumis sativus*, *Pisum sativum*, and *Vigna radiata*. The observed substantial antimicrobial activity rates have been linked with the ROS and RNS identified herein. Notably, the applied APPJ treatment did not impede seed germination, seed coat integrity, or early plant growth, proving the high potential of the CAPP-based disinfection techniques for agricultural practice. Future implementation of the efficient, innovative CAPP-based solutions into the seed production pipelines may contribute to reducing crop losses in addition to limiting the occurrence and spread of economically significant bacterial plant pathogens.

5. Patents

The construction of the utilized portable plasma jet and the application of the described plasma-based system for the eradication of plant pathogenic bacteria are protected by Polish Patent Applications, nos. P.429275 and P.438360, respectively.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/app15031255/s1, Table S1. Germination indices for plant seeds treated with the APPJ, which operated under the optimal working parameters, in contrast to the non-treated controls.

Author Contributions: J.O., W.S., P.J., A.D. and A.M.-P. handled conceptualization; A.D. and P.J., tailored the APPJ system for agricultural application; P.P. conducted multivariate optimization of the plasma-generating parameters; P.J. obtained and interpreted the OES spectra; D.T. and P.J. participated in the determination of the plasma-liquid interactions, revealed the RNS-related mechanisms, and described these results. Also J.O. participated in OES acquisition and conducting the colorimetric assays. W.S. and A.M.-P. were responsible for selection of experimental methodology for bacterial and seed-involving tests. J.O. conducted all the biological experiments. J.O. statistically evaluated and visualized the obtained biological data. J.O. described the collected results and discussed them with the literature. W.S. was granted funding for this study and administered this project. J.O. wrote the first version of the presented manuscript. A.M.-P., A.D., P.J. and E.L. corrected the manuscript.

P.P., E.L., P.J., A.D., D.T., W.S. and A.M.-P. supervised the experimental work of J.O., took part in discussion, and participated in preparation of the final form of the manuscript. All authors have read and agreed to the published version of the manuscript.

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