Received: 8 October 2017 | Accepted: 8 February 2018
DOI: 10.1002/bit.26565

ARTICLE

WILEY BIOTECHNOLOGY & BIOENGINEERING

Rapid eradication of bacterial phytopathogens by atmospheric pressure glow discharge generated in contact with a flowing liquid cathode

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Funding information
Ministerstwo Nauki i Szkolnictwa Wyższego, Grant numbers: 530-M031-D567-16-1A, D530-M031-D712-17, W3-Z16; Narodowe Centrum Nauki, Grant numbers: 2014/13/B/ST4/05013, 2016/21/N/NZ1/02783

Abstract

Diseases caused by phytopathogenic bacteria are responsible for significant economic losses, and these bacteria spread through diverse pathways including waterways and industrial wastes. It is therefore of high interest to develop potent methods for their eradication. Here, antibacterial properties of direct current atmospheric pressure glow discharge (dc-APGD) generated in contact with flowing bacterial suspensions were examined against five species of phytopathogens. Complete eradication of *Clavibacter michiganensis* subsp. *sepedonicus*, *Dickeya solani*, and *Xanthomonas campestris* pv. *campestris* from suspensions of $OD_{600} \approx 0.1$ was observed, while there was at least 3.43 logarithmic reduction in population densities of *Pectobacterium atrosepticum* and *Pectobacterium carotovorum* subsp. *carotovorum*. Analysis of plasma-chemical parameters of the dc-APGD system revealed its high rotational temperatures of 2,300 ± 100 K and 4,200 ± 200 K, as measured from N_2 and OH molecular bands, respectively, electron temperature of 6,050 ± 400 K, vibrational temperature of 4000 ± 300 K, and high electron number density of 1.1×10^{15} cm^{-1}. In addition, plasma treatment led to formation of numerous reactive species and states in the treated liquid, including reactive nitrogen and oxygen species such as NO_x, NH, H_2O_2, O_2, O, and OH. Further examination revealed that bactericidal activity of dc-APGD was primarily due to presence of these reactive species as well as to UVA, UVB, and UVC irradiation generated by the dc-APGD source. Plasma treatment also resulted in an increase in temperature (from 24.2 to 40.2 °C) and pH (from 6.0 to 10.8) of bacterial suspensions, although these changes had minor effects on cell viability. All results suggest that the newly developed dc-APGD-based system can be successfully implemented as a simple, rapid, efficient, and cost-effective disinfection method for liquids originating from different industrial and agricultural settings.

KEYWORDS
Clavibacter sp, *Dickeya* sp, disinfection, *Pectobacterium* sp, plasma-liquid interactions, *Xanthomonas* sp

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1 | INTRODUCTION

According to the United Nations, human population is estimated to exceed 9.7 billion people worldwide by 2050 and so provision of sufficient amounts of high quality nutritious food will be one of the main challenges of the 21st century (World Population Prospects, 2015). Significant crop losses result from a wide spread of over 50,000 plant pathogens, including fungi, viruses, bacteria, and nematodes (Fletcher et al., 2006). As neither breeding for resistance, genetic modifications, implementation of plant growth promoting rhizo bacteria (PGPR) (diCenzo, Zamani, Milunovic, & Finan, 2016; Zamani, DiCenzo, Milunovic, & Finan, 2017), nor physical or chemical treatments have succeeded so far, the main strategy undertaken for control of plant pathogenic bacteria involves preventive measures, with special regards to planting pathogen-free certified seeds, performing inspection of fields during vegetation, implementing crop rotation, as well as following hygienic procedures during harvest, post-harvest, and storage (Czajkowski, Pérombelon, van Veen, & van der Wolf, 2011). In this context, effortless and effective elimination of plant pathogens of high economic importance (Mansfield et al., 2012), in our case *Clavibacter michiganensis* subsp. *sepedonicus* (Cms), *Dickeya* spp., *Pectobacterium* spp., and *Xanthomonas campestris* pv. *campestris* (Xcc), from crop production pipelines and generated wastes is of high concern. Such new eradication methods can provide a highly attractive green alternative to the use of toxic disinfecting agents often utilized within industrial and agricultural settings.

Cms, often mentioned as one of the most devastating bacterial plant pathogens, is a Gram(+) pleomorphic actinomycete (Wolf Van der, Elphinstone, Stead, & Metzler, 2005). This microorganism causes bacterial ring rot (BRR) on potatoes and is listed among the A2 quarantine pests by the European and Mediterranean Plant Protection Organization (EPPO) (Smith, McNamara, Scott, & Holderness, 1997). Additionally, Zero Tolerance regulation was introduced to seed potato certification programs in order to limit its spread (de Boer & Slack, 1984). Highly infectious BRR can be marginal in one growing season but eventually leads to even 60% incidence in the subsequent one (Baribeau, 1948). Economic losses that arise from the presence of Cms result mainly from potato seed rejections and were estimated to vary in 13 US states from 0 to 15%, with mean of 3.6% per seed acreage (de Boer, 1987).

Bacteria from the genera *Dickeya* and *Pectobacterium* used to be classified together within the *Erwinia* genus, but advances in the identification methods resulted in separation of these taxa (Hauben et al., 1998; Samson et al., 2005). *Dickeya* and *Pectobacterium* spp. cause undistinguishable disease symptoms, that is, soft rot in the field, storage, or transportation on broad range of crops, vegetables, and ornamentals, in addition to blackleg restricted solely to potato plants during vegetation (Perombelon, 1992). Perombelon and Kelman (1980) estimated the financial impact of these plant pathogens as $50-100 million annually, and subsequent studies confirmed widespread presence of *Dickeya* and *Pectobacterium* spp. both in fields and storage facilities (Potrykus et al., 2016; Toth et al., 2011; Tsror et al., 2009).

Another bacterial phytopathogen of interest is Xcc, the etiological agent of black rot of crucifers (Williams, 1980). Development of black rot depends on environmental conditions coupled with factors of the host genus, species, cultivar, and age (Swings & Civerolo, 1993). Although Xcc is considered a seed-borne pathogen, it is also capable of infecting plants through leaf stomata, hydathodes, wounds, and roots (Mishra & Arora, 2012). Economic losses resulting from yield reduction can reach approx. 50%, and 50–70% for cabbage and cauliflower, respectively (Jorgensen & Walter, 1955).

Up to now, numerous sterilization and disinfection methods involving flame, dry or moist heat, filtration, and irradiation, or different chemicals, that is, hypochlorites, acids, quaternary ammonium compounds, ozone, hydrogen peroxide, chlorine dioxide, acidified sodium chlorite, trisodium phosphate, iodine, bromine, ethylene oxide, formaldehyde, propylene oxide, have been utilized against microorganisms (Parish et al., 2003). Also other physical (hot water, dry hot air, steam, solar, and UV irradiation), chemical (essential oils, antibiotics, natural bactericides like caffeine or chitosan, synthetic antimicrobial peptides) and biological (bacteriophages, bacterial symbionts, and PGPR) measures have been tested against plant pathogenic bacteria (Czajkowski et al., 2011). Nonetheless, the number of disinfection procedures commonly utilized in agriculture and industrial processing pipelines is rather limited that explains arising demand for other more environmentally-friendly and cost-effective approaches. Such measures could involve atmospheric pressure plasma (APP) sources of antimicrobial properties due to generated electrons, ions, heat, UV light, electric field, and various active species, and radicals (Ikawa, Kitano, & Hamaguchi, 2010). APP can be produced with the aid of various sources, including dielectric barrier discharges (DBDs) (Butscher, Van Loon, Waskow, Rudolf von Rohr, & Schuppler, 2016; Lu, Liu, Song, Zhou, & Niu, 2014; Miao & Yun, 2011; Shen et al., 2015; Tiede, Hirschberg, Viöl, & Emmert, 2016; Xiong, Roe, Grammer, & Graves, 2016; Yong et al., 2015), corona discharges (Kuwahara, Kuroki, Yoshida, Saeki, & Okubo, 2012; Masaoka, 2007), radio frequency discharges and plasmas (Akitsu, Ohkawa, Tsuji, Kimura, & Kogoma, 2005; Laroussi et al., 1999; Li et al., 2008; Matan, Puangjinda, Phothisuwan, & Nisoa, 2015; Ohkawa et al., 2006), microwave discharges (Gabriel et al., 2016; Park et al., 2007), and glow discharges (GD) (Dzimitrowicz et al., 2016a,b; Ikawa et al., 2010). To the best of our knowledge, DBD (Lu et al., 2014; Tiede et al., 2016; Xiong et al., 2016) and radio frequency discharges and plasmas (Laroussi et al., 1999; Ohkawa et al., 2006) have been implemented for microbiological and biomedical applications so far. By replacing DBD with GD, plasma-chemical parameters, for example, rotational temperature (T_{rot}) vibrational temperature (T_{vib}) of diatomic molecules, excitation temperature of atoms (T_{exc}), electron number density (n_e), and concentrations of different reactive species could be even enhanced. As a result, type of generated radicals and reactive species could be advantageously changed, leading to higher efficiency of the microbial inactivation process.

By now, direct effects of APPs have been mainly examined toward microorganisms responsible for diseases in humans, for example, *Escherichia coli*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa* (Gabriel et al., 2016; Miao & Yun, 2011; Shen et al., 2015) in addition to

microbial contaminants of food products, for example, *Listeria monocytogenes*, *E. coli*, and *Salmonella* spp. (Fernández & Thompson, 2012; Kim et al., 2011). Also plasma-treated water (PTW), obtained by exposure of de-ionized water to a low-temperature plasma jet generated under He atmosphere, was proposed as a promising inactivation method for *E. coli* in an indirect multi-step procedure (Ikawa, Tani, Nakashima, & Kitano, 2016).

Notably, no flow-through APP-based system providing continuous exposure of plant pathogenic microorganisms to plasma-derived sterilizing agents, neither has been proposed nor developed. For this reason, a flow-through plasma-reaction system with direct current atmospheric pressure glow discharge (dc-APGD) generated in contact with flowing bacterial suspensions acting as the liquid cathode was reported here. In terms of phytopathogens, to the best of our knowledge, only a plant pathogenic fungus *Cladosporium fulvum* has been subjected to APP treatment so far (Lu et al., 2014). Therefore antibacterial properties of this system against five worldwide present plant pathogenic bacteria, namely Cms, *Dickeya solani* (Dsol), *Pectobacterium atrosepticum* (Pba), *Pectobacterium carotovorum* subsp. *carotovorum* (Pcc) and Xcc, were shown here for the first time. The proposed plasma-reaction system allowed for much higher throughput of the input liquids, hence, the enhanced inactivation rate of mentioned bacterial pathogens in a more convenient way, that is, a direct and one-step process. The mechanism of bacterial eradication was additionally discussed, focusing on plasma-chemical parameters determined for gas and liquid phases of dc-APGD. Due to superior spectroscopic characteristics and direct impact on phytopathogenic microorganisms, it was supposed that future applications of the flow-through dc-APGD plasma-reaction system within industrial and agricultural settings could result in a substantial decrease of the economic losses related to presence and spread of plant pathogenic bacteria.

2 | MATERIALS AND METHODS

2.1 | dc-APGD plasma-reaction system

For efficient inactivation of phytopathogenic bacteria, a flow-through plasma-reaction system based on low power dc-APGD generated in contact with flowing bacterial suspension acting as the liquid cathode was developed (Figure 1). Initially, operating parameters of the plasma-reaction system, that is, flow rate of the liquid cathode solution, discharge gap, discharge current, and discharge voltage, were selected to provide stable operation of dc-APGD generated in contact with a sterile 0.85% NaCl solution. A NaCl solution or a bacterial suspension (A) was delivered by a four-channel peristaltic pump (B, MasterFlex L/S, Cole-Parmer®, Vernon Hill, IL) to a quartz plasma-reaction chamber (C) through a quartz capillary (D, ID ~ 2.0 mm), onto which a graphite tube (E, ID ~ 4.0 mm) was mounted. Flow rate of the 0.85% NaCl solution or bacterial suspension was 3.5 mL min^{-1}. dc-APGD (F) was sustained in open-to-air atmosphere (no additional gas like Ar or He was required) within a 5-mm gap between a pin-type tungsten electrode (G), acting as the anode, and surface of the flowing 0.85% NaCl solution or bacterial suspension, acting as the liquid cathode. To provide electrical contact, a

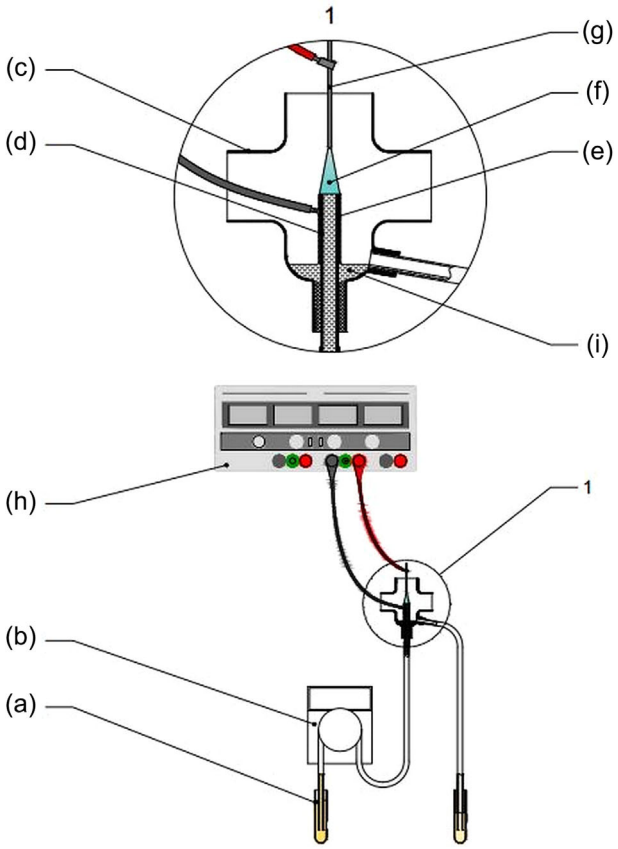

FIGURE 1 A schematic illustration of the dc-APGD-based flow-through system developed for rapid eradication of bacterial phytopathogens. Bacterial suspension (a) is pumped into the system (enlarged in the circle on the top and labeled as 1), where the eradication process takes place. (a) bacterial suspension, (b) peristaltic pump, (c) quartz chamber, (d) quartz capillary, (e) graphite tube, (f) dc-APGD, (g) pin-type tungsten electrode, (h) dc-HV generator, (i) solution after the dc-APGD treatment

Pt wire was attached to the graphite tube. To charge the anode at ~1100 V, a direct current high voltage generator (H, dc-HV, Dora Electronics Equipment, Wroclaw, Poland) was used. Discharge current of 50 mA was stabilized by a 10 kΩ ballast resistor (Tyco Electronics, Berwyn, PA). The power input of the system was estimated as 262 Wh L^{-1}. After passing through the developed dc-APGD plasma-reaction system, the plasma-treated 0.85% NaCl solution or bacterial suspension (I) was collected into a sterile vial with a stopper.

2.2 | The plasma-chemical properties of dc-APGD

To evaluate plasma-chemical properties of dc-APGD generated in contact with the flowing liquid cathode, optical emission spectrometry (OES) measurements of the gas phase of the discharge (during dc-APGD treatment of the 0.85% NaCl solution) and UV/Vis spectrophotometry measurements of plasma-treated solutions and bacterial suspensions were performed. Since the gas phase of the discharge was not uniform, while the reactive region was relatively thin as compared to the whole discharge gap, radiation emitted by the near-cathode

zone (up to ~2 mm from the edge of the graphite tube) of dc-APGD was 1:1 imaged on the entrance slit (10 μm) of a Shamrock SR-500i (Andor, United Kingdom) spectrometer equipped with a Newton DU-920P-OE CCD camera, using an achromatic quartz lens ($f = 60$). Acquisition time of the CCD camera was 0.50 s. Spectroscopic parameters of plasma in dc-APGD, that is, $T_{rot}(N_2)$, $T_{rot}(OH)$, $T_{vib}(N_2)$, $T_{exc}(H)$, and n_e, were evaluated by analyzing rotational-vibrational bands of OH and N_2 molecules and atomic lines of H. Non-resolved emission bands of N_2 (C-B system, 0–2 transition with band head at 380.2 nm), and OH (A-X system, 0–0 transition with band head at 308.9 nm), were selected for evaluation of $T_{rot}(N_2)$ and $T_{rot}(OH)$, respectively. Measured experimental structures of N_2 and OH bands were compared with spectra of these molecules simulated at various T_{rot} values, using SpecAir and Lifbase programs, respectively. The fitting procedure with minimization of the chi-square (χ^2) parameter was applied (Greda, Swiderski, Jamroz, & Pohl, 2016). Uncertainty of temperature measurements, expressed as relative standard deviation (RSD), was below 10%. $T_{vib}(N_2)$ was determined by the Boltzmann plot method; four transitions of the ($C^3\Pi_u$-$B^3\Pi_g$) N_2 band, that is, (0–2), (1–3), (2–4), and (3–5), were used (Tiede et al., 2016). $T_{exc}(H)$ was calculated using the two-line intensity method for H_α (656.3 nm) and H_β (486.1 nm) atomic lines (Jamróz & Żyrnicki, 2011). n_e (in cm^{-3}) was determined by applying the Stark broadening effect for the H_β line (Gigosos, González, & Cardeñoso, 2003).

The concentration of H_2O_2 was determined on the basis of peroxovanadium cation (VO_3^{2+}) formation through reaction of H_2O_2 with ammonium metavanadate (NH_4VO_3) in acidic medium, and spectrophotometric measurements at λ = 450 nm (Jamróz & Żyrnicki, 2011). The H_2O_2 concentration was assessed in a 0.85% NaCl solution before and after 10, 20, 40, and 60 min from dc-APGD treatment of this solution. All spectrophotometric measurements were performed using a double-beam UV/Vis Specord 210 Plus (Analytik Jena AG, Jena, Germany) instrument. To determine changes in temperature and pH of the 0.85% NaCl solution before and after 10, 20, 40, and 60 min from dc-APGD treatment, a CPC–505 pH/conductivity-meter (Elmetron, Zabrze, Poland) equipped with a temperature sensor was used.

2.3 | Bacterial strains, cultures, and suspensions

Strains of bacterial phytopathogens used in this study and their main characteristics are listed in Table 1. All utilized biological material originated from collection of plant pathogenic bacteria of the Intercollegiate Faculty of Biotechnology University of Gdansk and Medical University of Gdansk (IFB UG & MUG). Bacterial phytopathogens were recovered from frozen stocks kept at −80 °C in 40% glycerol. Dsol, Pcc, and Pba were cultured in TSB (BTL, Poland), while Xcc and Cms were grown in GF (Agarwal, Mortensen, & Mathur, 1989) or NCP-88 (Cruz, Wiese, & Schaad, 1992) media, respectively. Solid media were prepared by supplementing the corresponding liquid media with 15 g L^{-1} of agar. Bacteria were grown at 28 °C.

In order to prepare bacterial suspensions, overnight liquid cultures of investigated bacterial strains were centrifuged (6,500 rpm, 10 min) to remove supernatants and harvest the cells. The obtained cell pellets were washed twice with a sterile 0.85% NaCl solution to get rid of growth media residuals. All bacterial suspensions were subsequently adjusted to optical density $OD_{600} \approx 0.1$ (approx. 1×10^8 colony forming units per ml (CFU ml^{-1}) except for Cms; see Table 2) in the isosmotic sterile 0.85% NaCl solution.

2.4 | Eradication of bacterial phytopathogens by dc-APGD

A total of 3.5 ml of bacterial phytopathogens suspensions (at $OD_{600} \approx 0.1$) in 0.85% NaCl was introduced to the dc-APGD-based plasma-reactor system (Figure 1), which operating conditions were optimized. After passing through the above-mentioned system, plasma-treated suspensions were gathered into sterile polypropylene vials for further analysis. For each strain, the experiment was carried out in three repetitions. To ensure that the developed dc-APGD-based plasma-reaction system was free from any biological contaminants, all its components in contact with introduced bacterial suspensions were sterilized with 70% (v/v) ethanol solution before use. Each time, the ethanol solution was passed through the system for several minutes and removed from the liquid cathode compartment. Then, its remnants were washed out with excessive amount of the sterile 0.85% NaCl solution. Additionally, a negative control sample containing sterile 0.85% NaCl was introduced to the dc-APGD-based plasma-reaction system to exclude any incidental bacterial contamination. Another control sample was used to ensure effective removal of ethanol solution residuals. It was prepared by introduction of bacterial suspensions to the system without the discharge running.

Assessment of antibacterial activity of dc-APGD treatment against phytopathogens was performed using the standard serial dilution plating method. Bacterial suspensions were serially diluted up to 10^{-6} prior to introduction of $OD_{600} \approx 0.1$ suspensions to the dc-APGD-based plasma-reaction system. Hundred microliter of each dilution and the concentrated bacterial suspension was plated on appropriate solid growth medium. dc-APGD-treated bacterial suspensions were collected, serially diluted up to 10^{-6} and 100 μl of each dilution and the concentrated suspension was plated on appropriate growth medium. Afterwards, plates were incubated for 48 hr at 28 °C, following which bacterial colonies were counted. Efficiency of plasma treatment was depicted as logarithmic reduction (or inactivation of the microorganisms was defined as complete) in addition to percentage reduction in the amount of viable cells (CFU ml^{-1}) in plasma-treated suspensions compared to non-plasma-treated controls. Effectiveness of dc-APGD was compared to that of UVC irradiation generated by two TUV30W G30T8 (Philips, The Netherlands) lamps with max wavelengths at 253.7 nm.

2.5 | Effects of temperature and pH changes on bacterial viability

In order to state which properties of dc-APGD contributed to observed antibacterial properties of the generated discharge, $OD_{600} \approx 0.1$ bacterial suspensions were subjected to changes in temperature and pH. As pH of the plasma-treated 0.85% NaCl solution was determined to change from

TABLE 1 Bacterial phytopathogens used in the present study and their main characteristics

Bacterial species	Strain IFB UG & MUG no.	Strain nos. in other collections[a]	Country of isolation	Host	Disease caused
Clavibacter michiganensis subsp. *sepedonicus* (Cms)	IFB 9034	CL 0638	Poland, 2010	*Solanum tuberosum*	Potato ring rot
Dickeya solani (Dsol)	IFB 0099	IPO 2276, LMG 28824	Poland, 2005	*Solanum tuberosum*	Blackleg and soft rot
Pectobacterium atrosepticum (Pba)	IFB 5103	SCRI 1086	Canada, 1985	*Solanum tuberosum*	Blackleg and soft rot
Pectobacterium carotovorum subsp. *carotovorum* (Pcc)	IFB 5118	SCRI 136	USA	*Solanum tuberosum*	Blackleg and soft rot
Xanthomonas campestris pv. *campestris* (Xcc)	IFB 9022	LMG 582	Belgium, 1980	*Brassica* sp.	Black rot

[a]LMG BCCM/LMG, Belgian Coordinated Collections of Microorganisms (Gent, Belgium); IPO, Plant Research International (Wageningen, The Netherlands); SCRI, Scottish Crop Research Institute Collection (Dundee, Scotland); CL, Central Laboratory of Main Inspectorate of Plant Health and Seed Inspection (Torun, Poland); UG & MUG, University of Gdansk and Medical University of Gdansk.

6.0 to 10.8, the effect of 1 min and 20 min incubation time in these conditions on survival of bacterial cells was investigated. As plasma-treated liquids were also observed to heat up to 40 °C when the discharge was running, the effect of exposure of bacterial suspensions to 40 °C in comparison to 60 °C, for 1 min and 20 min, was examined as well. One minute subjection corresponded to the time when bacteria were in contact with the discharge, while 20 min referred to the time when phytopathogens were exposed to contents of the dc-APGD-treated liquid before having been plated to solid media. The remaining amount of viable bacterial cells (CFU ml^{-1}) after pH or thermal treatment was depicted in comparison to non-treated controls.

2.6 | Statistical analysis

Statistical significance of the observed differences in the cell viability was determined with R packages (Gentleman, Huber, & Carey, 2011).

TABLE 2 Efficiency of dc-APGD treatment against plant pathogenic bacteria

Repetition	Initial amount of bacterial cells [CFU mL^{-1}]	Amount of bacterial cells after the plasma treatment [CFU mL^{-1}]	Logarithmic reduction
Clavibacter michiganensis subsp. *sepedonicus* (Cms)			
I	9.00×10^3	0	Complete
II	2.90×10^3	0	Complete
III	3.60×10^3	0	Complete
Dickeya solani (Dsol)			
I	6.90×10^7	0	Complete
II	1.51×10^8	0	Complete
III	1.25×10^8	0	Complete
Pectobacterium atrosepticum (Pba)			
I	2.28×10^7	0	Complete
II	2.84×10^7	5.91×10^3	3.68
III	1.70×10^7	0	Complete
Pectobacterium carotovorum subsp. *carotovorum* (Pcc)			
I	2.80×10^7	7.76×10^3	3.56
II	2.81×10^7	0	Complete
III	3.20×10^7	1.2×10^4	3.43
Xanthomonas campestris pv. *campestris* (Xcc)			
I	2.87×10^7	0	Complete
II	2.98×10^7	0	Complete
III	3.17×10^7	0	Complete

Normality of variances was examined by the Shapiro-Wilk test (Shapiro & Wilk, 1965). Equality of variances was evaluated by the Levene's test (Levene, 1960). For comparing susceptibility of various phytopathogens to dc-APGD and to UV treatment, as ANOVA requirements were not fulfilled, analysis of variance was performed with the non-parametric Kruskal-Wallis test (Kruskal & Wallis, 1952). If statistically significant differences between groups were noted, the post-hoc test using the Fisher's least significant difference criterion from the R agricolae package was applied. In order to assess the impact of pH, and temperature changes on viability of cells, two-sided *t*-test (Student's or Welch's *t*-test depending on equality of variances) was used to compare plasma-treated groups to the corresponding controls. $p < 0.05$ was used for all calculations.

3 | RESULTS AND DISCUSSION

3.1 | Efficiency of dc-APGD against bacterial phytopathogens

It was established that dc-APGD generated in contact with the flowing liquid cathode applied in the developed plasma-reaction system was a potent method for eradicating phytopathogens. Logarithmic reduction in densities of plant pathogenic bacterial populations ranged from 3.43 up to complete eradication of viable bacterial cells from plasma-treated suspensions (see Table 2). Of the species examined, Cms, Dsol, and Xcc were determined to be the most susceptible to eradication by the applied method, with no viable cells detected following the plasma treatment. Pcc and Pba were more resistant as shown in Figure 2; however, no statistically significant differences between the species were noted (at *p*-value <0.05). This is in contrast to observations of Ermolaeva et al. (2011), who suggested that Gram(−) bacteria are less resistant to plasma than Gram(+) bacteria. In the present work, Gram(+) Cms was completely inactivated by dc-APGD, while Gram(−) Pcc and Pba were found to be more resistant to applied treatment.

To the best of our knowledge, no dc-APGD-based plasma-reaction system working in a flow-through mode has been proposed until now for eradication of bacterial phytopathogens. Former applications of APPs sources mainly involved eradication of human pathogens or microbial contaminants of food present on surfaces of Petri and quartz plates, seeds, plastic bottles, animal skin, or model materials imitating, for example, nails or hoofs (Butscher et al., 2016; Ermolaeva et al., 2011; Gabriel et al., 2016; Lu et al., 2014; Masaoka, 2007; Xiong et al., 2016). According to our best knowledge, Lu et al. (2014) were the first to use an APP source, in this case DBD, to inactivate with nearly 100% efficacy a plant pathogenic fungus *Cladosporium fulvum*. An atmospheric cold plasma jet was also utilized for direct curing of *Philodendron erubescens* leaf surfaces from fungal diseases (Zhang et al., 2014). In our opinion, elimination of microorganisms from intractable suspensions is innovative and worthy of special attention as ions and electrons are efficiently absorbed by liquids and could not directly interact with treated bacteria, which makes development of an effective sterilizing system particularly challenging (Ikawa et al., 2010). Despite these constraints, data reported here show that the described dc-APGD plasma-reaction system can efficiently disinfect highly contaminated liquid suspensions.

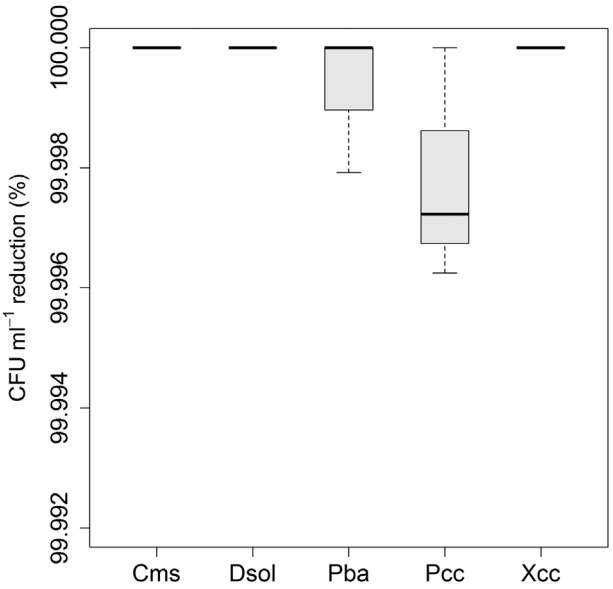

FIGURE 2 Comparison of susceptibility of phytopathogens from diverse species to dc-APGD treatment. Percentage reduction in the amount of viable bacterial cells (CFU ml^{-1}) achieved in the applied dc-APGD-based flow-through plasma-reaction system is shown on the y axis. Plant pathogens tested are depicted on the x axis: Cms, *Clavibacter michiganensis* subsp. *sepedonicus*; Dsol, *Dickeya solani*; Pba, *Pectobacterium atrosepticum*; Pcc, *Pectobacterium carotovorum* subsp. *carotovorum*; Xcc, *Xanthomonas campestris* pv. *campestris*. Lower and upper quartiles are enclosed in the box, the bar represents the median, while whiskers are minimal and maximal values. No statistically significant differences between groups were noted with Kruskal-Wallis test at $p < 0.05$

Effectiveness of bacterial inactivation by APPs differs between previous studies, which is most likely due to variation in utilized plasma sources, plasma parameters, eradicated microbial species, plasma exposition time, and experimental setups. For instance, only a 2-logarithmic reduction value was achieved following 40 min exposition of *S. aureus* to a dc gas-liquid phase Ar APP (Shen et al., 2015). On the other hand, approximately 6-logarithmic reduction was observed after 20-min plasma treatment of spore-forming indicators *Bacillus atrophaeus* and *Geobacillus stearothermophilus* placed on a cellulose carrier (Akitsu et al., 2005). However, non-sporulating microorganisms like *E. coli*, *Salmonella enteritidis*, and *Candida albicans*, were inactivated within 1-min exposure (Akitsu et al., 2005). In the present study, efficacy of 3.43 logarithmic reduction up to complete eradication within approx. One minute of dc-APGD exposure was achieved, combining optimal exposure time with sufficient potency in comparison to APPs systems cited above.

As UV irradiation being the gold standard in liquid sterilization showed 99.996 to 100% reduction in the amount of viable cells of tested phytopathogenic bacteria (Figure 3), it had similar effectiveness to the developed dc-APGD system. It is worth noting that potency of

1-min UV treatment was statistically different from that of 20-min exposure. Additionally, tested plant pathogens were unequally susceptible to UV irradiation, notably Pcc and Pba were the most resistant as was also observed in case of applied dc-APGD treatment.

3.2 | Mechanism of phytopathogens inactivation

3.2.1 | Effect of dc-APGD on solution pH and its role in bacterial viability

By evaluating antibacterial effects of plasma-liquid interactions on *E. coli*, Ikawa et al. (2010) hypothesized that pH was a critical factor in mediating cell death as microbial inactivation was relatively ineffective when pH of treated bacterial suspensions exceeded 4.7. In the present study, the 0.85% NaCl solution following plasma treatment had alkaline pH 10.8 (60 min post dc-APGD treatment), compared to pH 6.0 determined before plasma treatment. During plasma treatment, surface of the flowing liquid cathode solution (consisting of 0.85% NaCl) is irradiated by positive ions (mostly ionized H_2O molecules) from the plasma anode giving rise to high probability that hydrated electrons (e^-_{aq}) are generated in the plasma-treated liquid ($H_2O^+ + H_2O = 2H_2O^+ + e^-_{aq}$) and other reactions take place (Tochikubo, Shimokawa, Shirai, & Uchida, 2014; Tochikubo, Shirai, Uchida, et al., 2014). The resulting increase in pH of the 0.85% NaCl solution after dc-APGD treatment might be associated with generation of OH^- ions via reaction of e^-_{aq} with H_2O molecules ($2H_2O + 2e_{aq}^- = H_2 + 2OH^-$). In addition, some of e^-_{aq} might have recombined by reverse reactions. Remaining water cations could interact with surrounding H_2O molecules, producing hydronium cations (H_3O^+) and OH radicals ($H_2O^+ + H_2O = H_3O^+ + OH$). Recombination of H_3O^+ molecules and e^-_{aq} could then generate H atoms ($H_3O^+ + e^-_{aq} + = H + H_2O$). It was supposed that when the plasma anode and the solution cathode would be separated, that is, as in U-shaped electrochemical cells described by Tochikubo, Shimokawa, et al. (2014), Tochikubo, Shirai, et al. (2014) or Wang et al. (2014), there would be observed an increase of pH from ~6–7 to 10 in the catholyte solution and a decrease of pH from ~6–7 to 5 in the anolyte solution. In case of the plasma-reaction system developed in the present work, plasma anode and solution cathode compartments were not separated from each other. Therefore, pH measured was a result of two different processes, one leading to generation of the OH^- ions while the other being responsible for generation of the H_3O^+ ions. As was established here, dc-APGD-treated solutions (bacterial suspensions in 0.85% NaCl) were alkaline, pointing that reductive processes were dominating, that is, $2H_2O + 2e^-_{aq} = H_2 + 2OH^-$.

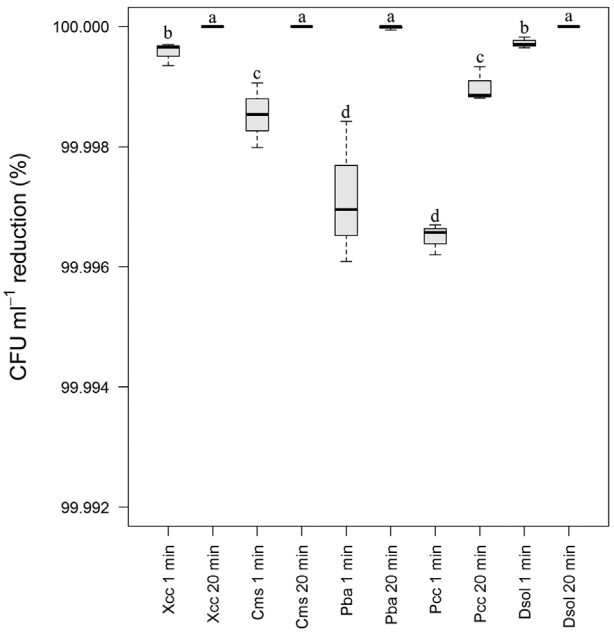

FIGURE 3 Comparison of susceptibility of phytopathogens from diverse species to UV light. Percentage reduction in the amount of viable bacterial cells (CFU ml^{-1}) is shown on the y axis. Tested plant pathogens together with time of treatment are depicted on x axis: Cms, *Clavibacter michiganensis* subsp. *sepedonicus*; Dsol, *Dickeya solani*; Pba, *Pectobacterium atrosepticum*; Pcc, *Pectobacterium carotovorum* subsp. *carotovorum*; Xcc, *Xanthomonas campestris* pv. *campestris*. Lower and upper quartiles are enclosed in the box, the bar represents the median, while whiskers are minimal and maximal values. Groups differencing in statistically significant manner are marked with diverse letters (Kruskal-Wallis test at $p < 0.05$ followed by post hoc using the Fisher's least significant difference criterion)

To determine contribution of pH variation to inactivation of tested phytopathogens, all bacteria were incubated for 1 min and 20 min in saline solutions at pH 6.0 or 10.8 (Figure 4). In comparison to non-incubated controls, there was a statistically significant decrease in amount of viable cells following 20 min incubation at pH 10.8 for Pba, Pcc, and Xcc. However, observed amounts of remaining viable phytopathogenic cells under these conditions are comparable to the ones noted for 1 min and 20 min incubations at pH 6.0 in addition to 1 min incubation at pH 10.8. Thus, it was concluded that pH changes generated by dc-APGD did not significantly influence survival of tested phytopathogens and, therefore, were not responsible for observed bactericidal properties. It needs to be stressed that elevated external pH is commonly encountered by phytopathogenic bacteria in their natural environment. Non-extremophiles, such as microorganisms investigated here, maintain pH 7.4–7.8 within cytoplasm mainly due to action of Na^+/H^+ antiporters as suggested by Padan, Bibi, Ito, and Krulwich, 2005. *Dickeya* and *Pectobacterium* spp. even trigger elevation of pH after having performed colonization of the plant, as they produce and secrete acetoin causing plant apoplast alkalization important for setting the pathogenic state of infection (Deochand, Mearman, & Grove, 2016). Also pectate lyases, which are main virulence factors of soft rot plant pathogens like Pba, Pcc, or Dsol, exhibit the highest efficacy at pH > 8.0.

3.2.2 | Production of reactive species by dc-APGD

OES was used to identify reactive species formed in the gas phase of dc-APGD plasma. Emitted radiation was recorded within the range of 200–900 nm (Figure 5). As can be seen, emission spectrum of dc-APGD generated in contact with the 0.85% NaCl solution mainly

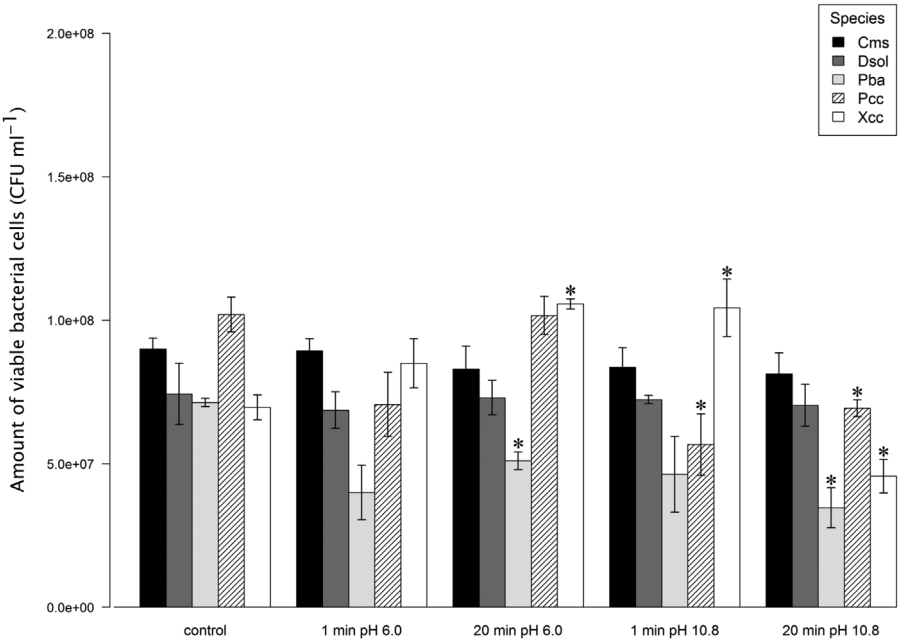

FIGURE 4 Comparison of susceptibility of phytopathogens from diverse species to pH generated by dc-APGD treatment. The amount of viable bacterial cells (CFU ml^{-1}) is shown on y axis, while time and type of pH treatment are depicted on x axis. Colors of bars represent the following bacterial species: black—*Clavibacter michiganensis* subsp. *sepedonicus* (Cms), dark gray—*Dickeya solani* (Dsol), light gray—*Pectobacterium atrosepticum* (Pba), striped—*Pectobacterium carotovorum* subsp. *carotovorum* (Pcc), white—*Xanthomonas campestris* pv. *campestris* (Xcc). Non-treated bacterial suspensions and bacterial suspensions incubated at pH 6.0 were included for comparison purposes. Means ± SE are depicted. Asterisks mark values differing in statically significant manner from the corresponding control (*t*-test at $p < 0.05$)

consisted of very intensive molecular bands of diatomic molecules such as NO, OH, N_2, and NH. In the UVC spectral region (<280 nm), numerous bands of NO molecules belonging to the γ-system ($A^2\Sigma^+$-$X^2\Pi$) were observed. Presence of NO molecules might result from reactions of N_2 molecules with O and H radicals, which were previously reported by Jamroz and Zyrnicki (2011). In the range of 280–400 nm, the most intense emission was established to originate from the (0–0) band of OH molecules belonging to the $A^2\Sigma$-$X^2\Pi$ system (with head at 309.4 nm) and (1–0), (0–0), and (0–1) bands of N_2 molecules belonging to the second positive $C^3\Pi_u$-$B^3\Pi_g$ system (with heads at 315.9, 337.1, and 357.7 nm, respectively). N_2 molecules might have originated from surrounding air, which is enriched in the discharge atmosphere (Jamróz, Gręda, Pohl, & Żyrnicki, 2014). A less intensive molecular band for OH molecules belonging to the $A^2\Sigma$-$X^2\Pi$ system was observed for (1–0) transition (with head at 286.0 nm). Intense (0–0) and (1–1) bands of NH molecules ($A^3\Pi$-$X^3\Sigma^-$ system) with heads at 336.0 nm and 337.0 nm, respectively, were also identified but they overlapped the (0–0) band of N_2 ($C^3\Pi$-$B^3\Pi$) molecules at 337.1 nm. Presence of NH molecules is also related to reaction between O and H radicals as was shown for NO molecules (Jamroz and Zyrnicki, 2011). In the spectral region >400 nm, intense atomic lines of H belonging to the Balmer series at 486.1 (H_β) and 656.2 nm (H_α), several less intensive N_2 bands of the second positive $C^3\Pi_u$-$B^3\Pi_g$ system (with heads at 399.8, 405.9, 427.0, and 434.4 nm for (0–3), (2–6), (1–5), and (0–4) transitions, respectively) and some weak N_2 bands of the $B^3\Pi_g$-$A^3\Sigma^+_u$ system (at 750.4, 654.48, 646.85 nm) were also identified. Atomic O lines (O I) at 777.2, 777.4, and 844.6 nm and weak ionic O lines (O II) at 441.5, 459.1, 459.6, 467.4, 466.6, and 470.7 nm were noted within this spectral range as well. In addition, extremely high emission from Na atoms in the near-cathode zone of dc-APGD was observed and corresponded to yellow illumination of the discharge. Accordingly, the most prominent Na atomic lines (Na I) were identified at 330.2, 417.7, 568.3, 589.6, 616.0, 781.0, 819.5, and 849 nm. Presence of Na atoms in the plasma zone was probably related

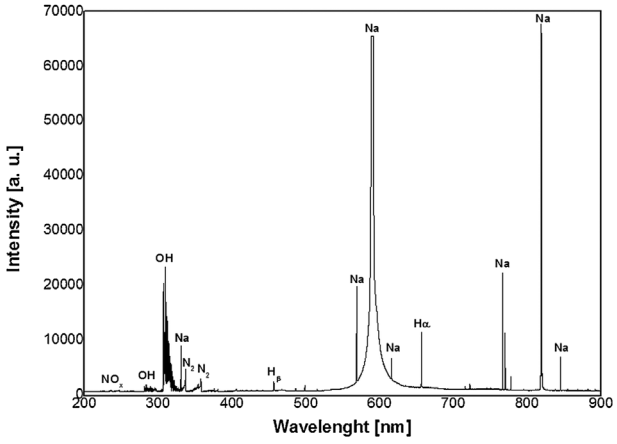

FIGURE 5 The emission spectrum of dc-APGD generated in contact with the flowing liquid cathode (0.85% NaCl)

to sputtering of bacterial NaCl suspensions. It is possible that gaseous Cl_2 was generated following dc-APGD treatment under these conditions (Tochikubo, Shimokawa, et al., 2014; Tochikubo, Shirai, et al., 2014). Cl_2 dissolved in water could further lead to production of ClO_x^- ($Cl_2 + H_2O^- = ClO^- + Cl^- + 2H^+$) as was shown by Wende et al. (2015). The other path for $ClOx^-$ generation is reaction $Cl^- + O = ClO^-$ (Wende et al., 2015). It is well known that chloride dioxide (ClO_2) is toxic to *Salmonella* spp., *E. coli*, and *L. monocytogenes* (Sy, Murrey, Harrison, & Beuchat, 2005). If formed, ClO_x^- could therefore contribute to antibacterial activity of dc-APGD. However, it was not examined whether ClO_x^- was present in plasma-treated suspensions, while Cl was not detected in acquired emission spectra of dc-APGD. Neither generation of superoxide anion radicals in the gas phase of the discharge was confirmed as the characteristic band system at 245 nm (Attri et al., 2015) was not identified in the emission spectrum of dc-APGD. Therefore, this species was not included in the discussion about the possible bacterial eradication mechanism.

Next, the concentration of H_2O_2 formed in bacterial suspensions treated by dc-APGD was measured. As this compound did not occur in any of untreated bacterial suspensions, all H_2O_2 molecules present in liquids after dc-APGD treatment resulted from plasma-liquid interactions occurring at the interfacial zone of the discharge and flowing bacterial suspensions. The concentration of H_2O_2 was estimated to be 92 mg L^{-1} and 103 mg L^{-1} when measured after 10 and 20 min, respectively, following dc-AGPD treatment. H_2O_2 might have originated from H_2O oxidative processes ($2H_2O = H_2O_2 + H_2$, $OH + OH = H_2O_2$) (Locke & Shih, 2011).

In line with results presented here, other groups have observed formation of H_2O_2 in the range of 1.8–38 mg L^{-1} following plasma treatment (Ivannikov, Lelevkin, Tokarev, & Yudanov, 2003). Plasma-liquid interactions also led to formation of such powerful RNS and ROS like NO_x, NH, N_2, O, and OH, as determined by OES measurements. It was presumed that all N and O-containing species identified in dc-APGD plasma could take part in reactions and processes responsible for further production of other ROS and RNS (Jamróz & Żyrnicki, 2011) resulting in oxidation of bacterial nucleic acids, proteins, and lipids (Juven & Pierson, 1996). As antibacterial activity of ROS and RNS is well-documented (Fang, 2004), observed eradication of plant pathogenic bacteria may be attributed to reactive species formed in the developed dc-APGD-based plasma-reaction system. Notably, ROS and RNS exposure triggers bacterial global oxidative stress response, which differs among various species. As reported by Jiang et al. (2016), for example, *Dickeya* spp. can reduce the impact of radicals through production of KatG and KatE catalases, AhpCF peroxidase, organic hydroperoxide resistance proteins, antioxidant pigment indigoidine, universal stress proteins UspA, UspB, UspE, and UspG, in addition to induction of DNA-damage-dependent SOS response proteins, that is, UvrA, UvrB, UvrD, RcN, RecAX, and RuvAB. Interestingly, of two genes encoding superoxide dismutases *sodA* and *sodC*, only the latter one is significantly upregulated upon exposure of *Dickeya* spp. to H_2O_2 (Jiang et al., 2016). Thus, observed differences in susceptibility of investigated bacterial plant pathogens to the applied dc-APGD system, although not statistically significant, could be related to variation in efficacy of their oxidative stress response systems.

3.2.3 | Generation of UV radiation by dc-APGD and its effect on bacterial viability

The following types of UV radiation were detected in the studied dc-APGD system (Figure 5): UVA (320–400 nm), UVB (280–320 nm), and UVC (<280 nm) (Weltmann et al., 2009) . In general, shorter wavelength photons exhibit higher energy and cause more damage to exposed microorganisms. UVA is poorly absorbed by microbial biomolecules, however, multiple ROS are generated via interaction of this UV radiation with intracellular chromophores (Pourzand & Tyrrell, 1999). UVB and UVC can directly affect DNA, presumably due to formation of cyclobutane pyrimidine dimers and pyrimidine(6–4) pyrimidinone photoproducts (Pfeifer, 1997), which if not repaired, lead to lethal blockage of transcription and DNA replication. However, as phytopathogens frequently inhabit plant phyllosphere, they are exposed to solar UV irradiation (95% UVA and 5% UVB, no UVC), which has led to evolution of several mechanisms increasing their overall fitness. In addition to the UVA-dependent oxidative stress response, bacteria often possess protective pigmentation like carotenoids of *C. michiganensis* or xanthomonadin produced by *X. campestris* (Jacobs, Carroll, & Sundin, 2005). Considering that greater than 4.4 logarithmic reduction in viable cells of tested plant pathogenic bacteria was observed post exposition to UV irradiation (percentage reductions in population densities ranged from 99.996 to 100%; Figure 3), this factor likely had important contribution to bacterial inactivation by dc-APGD.

3.2.4 | Determination of the plasma-chemical parameters

Plasma-chemical parameters of dc-APGD such as $T_{rot}(N_2)$, $T_{rot}(OH)$, $T_{vib}(N_2)$, $T_{exc}(H)$ as well as n_e were assessed to define plasma properties and its elementary processes. $T_{rot}(N_2)$ is related to kinetic plasma gas temperature (Greda et al., 2016). Based on fitting of the N_2 emission spectrum to the simulated vibration-rotational emission spectrum of this molecule, $T_{rot}(N_2)$ value at the plasma-liquid interfacial zone was estimated to be 2,300 ± 100 K, which is significantly higher than what has been reported for DBD-based plasma devices previously used for biological applications (Shen et al., 2015; Tiede et al., 2016). $T_{rot}(OH)$ (4,200 ± 200 K) and $T_{vib}(N_2)$ (4,000 ± 300 K) were quite similar and nearly two times higher than $T_{rot}(N_2)$, pointing a non-equilibrium state of dc-APGD plasma. $T_{exc}(H)$ was also elevated (6,050 ± 400 K), which was likely associated with existence of high energy electrons, relatively intense plasma excitation conditions, and efficient production of excited radicals and other reactive species (Shen et al., 2015). Finally, a relatively high value of n_e, that is, $(1.1 \pm 0.1) \times 10^{15}$ cm^{-1}, was also consistent with strong plasma excitation conditions within dc-APGD.

3.2.5 | Effect of dc-APGD on solution temperature and its role in bacterial viability

It was established that temperature of bacterial suspensions increased following dc-APGD treatment to 40.2 °C (10 min post dc-APGD

treatment) as compared to 24.2 °C before such treatment. The noted temperature change was in agreement with observations of Greda et al. (2016) on a similar flow-through system. Therefore, the effect of 1-min and 20-min exposure of tested bacteria to 40 °C and 60 °C was determined. Whereas complete eradication was observed following exposure to 60 °C for 20 min, exposure for the same time to 40 °C had little effect on survival of phytopathogens (Figure 6). As temperature resulting from plasma treatment roughly approximates maximum growth temperatures for investigated species, which ranges from 35 to 45 °C (Perombelon & Kelman, 1980), this factor did not contribute to antimicrobial properties of dc-APGD.

In summary, it appears that ROS, RNS, and UV radiation, readily produced in dc-APGD due to favorable conditions for plasma-chemical reactions and processes as reflected by outstanding values of T_{rot}, T_{vib}, T_{exc} and n_e, significantly contributed to antibacterial properties of the developed dc-APGD-based flow-through plasma-reaction system.

3.3 | Potential applications

The developed dc-APGD-based plasma-reaction system was shown to be an effective method for eradication of plant pathogenic bacteria, including Xcc, Cms, Pcc, Pba, and Dsol, from liquid suspensions. Both Gram(+) and Gram(−) species listed among the top ten plant pathogenic bacteria (Mansfield et al., 2012) were efficiently inactivated due to high yield of generated ROS, RNS, and UV radiation. Because this plasma-reaction system was working in a flow-through mode, suspensions of studied bacterial phytopathogens were continuously introduced through the graphite/quartz tube and overflowed it after plasma treatment. This treatment and interaction of different reactive components and UV radiation in gas and liquid phases at the interfacial zone had surface and near-surface character. However, bacterial suspensions were constantly replenished, leading to renewal of the surface available for action of mentioned species and radiation. Such a dynamic system was responsible for uniform treatment of bacterial suspensions. Certain variation in susceptibility of tested species to dc-APGD was observed, although in each case there was at least one repetition resulting in complete eradication of investigated species, including Pba and Pcc, whose inactivation rates, nonetheless, exceeded 3.43 logarithmic reduction values. Further optimization of the flow-through system is expected while scaling it up for industrial applications.

It was previously shown that improperly conducted agricultural engineering practices can contribute to spread of bacterial phytopathogens (Czajkowski et al., 2011; Perombelon & Kelman, 1980). In particular, harvesters not meeting hygienic standards, unsanitized storage areas, and inappropriately handled potentially infectious wastes could contribute to local spread of infectious materials. As the proposed dc-APGD-based plasma-reaction system was shown to be a potent device for disinfection of suspensions containing bacterial phytopathogens, its use for elimination of bacterial contaminants from industrial and agricultural wastewaters, liquid wastes from microbiological laboratories, and other putatively contaminated liquid disposals is highly possible and tends to be an attractive green alternative to existing

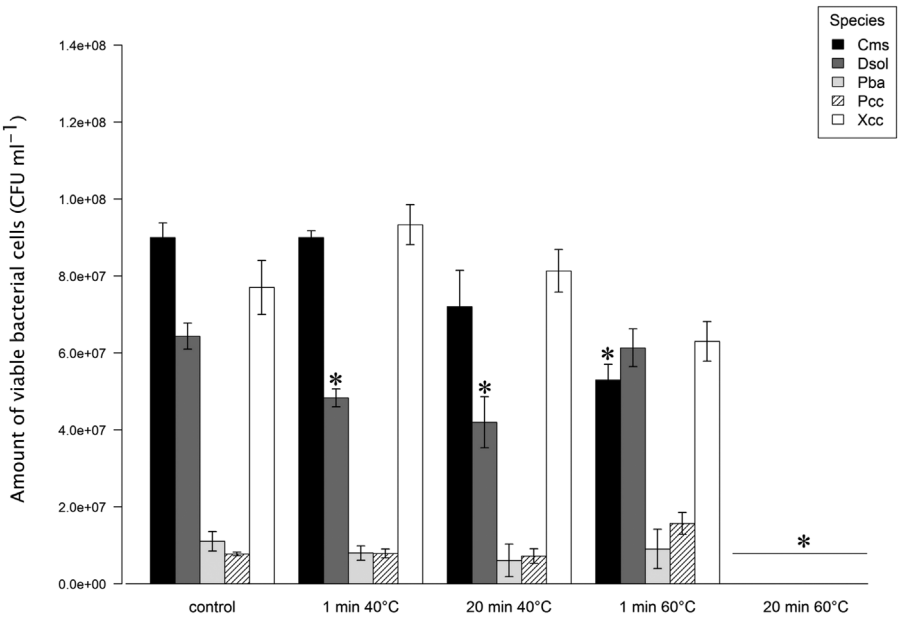

FIGURE 6 Comparison of susceptibility of phytopathogens from diverse species to temperature generated by dc-APGD treatment. The amount of viable bacterial cells (CFU mL^{-1}) is shown on y axis, while time and type of thermal treatment are depicted on x axis. Colors of bars represent following bacterial species: black—*Clavibacter michiganensis* subsp. *sepedonicus* (Cms), dark gray—*Dickeya solani* (Dsol), light gray—*Pectobacterium atrosepticum* (Pba), striped—*Pectobacterium carotovorum* subsp. *carotovorum* (Pcc), white —*Xanthomonas campestris* pv. *campestris* (Xcc). Non-treated bacterial suspensions and suspensions of plant pathogens subjected to 60 °C were included for comparison purposes. Means ± SE are depicted. Asterisks mark values differing in statically significant manner from the corresponding control (*t*-test at $p < 0.05$)

solutions. It is worth underlining that the described plasma-reaction system has very low power consumption (~55 W), basing on calculation on relation between applied voltage and discharge current.

APP-based treatment described in the present work is rapid, cost-effective, and does not involve highly irritating, toxic, and possibly carcinogenic substances, contrary to what is known for formaldehyde or chlorine compounds often applied for decontamination of infectious liquid laboratory-derived wastes (Council, 1989). Taking into account that dc-APGD can be used in purification of wastewaters containing, for example, Cr(VI) ions, organic dyes, and surfactants (Jamróz et al., 2014), the herein described method could also be applied for disposal of mixed wastes. As suggested by Shen et al. (2015), APP-treated liquids could retain antibacterial properties for prolonged time, facilitating further storage, transportation, or disposal of wastes. These advantages are also valid for the dc-APGD-based plasma-reaction system reported in this work.

ACKNOWLEDGMENTS

This work was financed by statutory activity subsidies from the Polish Ministry of Science and Higher Education for the Faculty of Chemistry of Wroclaw University of Science and Technology and Department of Biotechnology of IFB UG & MUG (530-M031-D567-16-1A and D530-M031-D712-17). AD, PJ, and PP are thankful to the National Science Centre (Poland) for co-financing scientific researches (research project no. 2014/13/B/ST4/05013). This work was also supported from the National Science Centre (Poland) via grant no. 2016/21/N/NZ1/02783 dedicated to AM.

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How to cite this article: Motyka A, Dzimitrowicz A, Jamroz P, Lojkowska E, Sledz W, Pohl P. Rapid eradication of bacterial phytopathogens by atmospheric pressure glow discharge generated in contact with a flowing liquid cathode. *Biotechnology and Bioengineering*. 2018;115:1581–1593. https://doi.org/10.1002/bit.26565