Effect of 222-nm krypton-chloride excilamp treatment on inactivation of *Escherichia coli* O157:H7 and *Salmonella* Typhimurium on alfalfa seeds and seed germination

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A R T I C L E   I N F O

A B S T R A C T

We examined the control effect of a 222-nm KrCl excilamp on foodborne pathogens on alfalfa seeds and compared it with a conventional 254-nm low-pressure (LP) Hg lamp. When the 222-nm KrCl excilamp treated seeds at 87, 174 and 261 mJ/cm^2, the log reductions of *Escherichia coli* O157:H7 (*E. coli* O157:H7) were 0.85, 1.77, and 2.77, respectively, and *Salmonella* Typhimurium (*S*. Typhimurium) experienced log reductions of 1.22, 2.27, and 3.04, respectively. When the 254-nm LP Hg lamp was applied at 87, 174, and 261 mJ/cm^2, the log reductions of *E. coli* O157: H7 were 0.7, 1.16, and 1.43, respectively, and those of *S*. Typhimurium were 0.75, 1.15, and 1.85, respectively. Therefore, it was shown that the 222-nm KrCl excilamp was more effective than the 254-nm LP Hg lamp in reducing foodborne pathogens. The germination rate decreased to less than 80% after 261 mJ/cm^2 treatment with the 254-nm LP Hg lamp, while more than 90% was maintained with 261 mJ/cm^2 222-nm KrCl excilamp treatment. DNA damage assay showed that the difference in germination rate was due to DNA damage resulting from 254-nm LP Hg lamp treatment. However, 222 nm KrCl excilamp treatment did not cause DNA damage, resulting in no difference in germination rate compared to that of non-treated alfalfa seeds. Overall, these results demonstrate the utility of the 222-nm KrCl excilamp as a foodborne pathogen control intervention for the alfalfa seed industry.

1. Introduction

Seed sprouts have been popular in recent years due to their nutritional value and low cost, compared to other fresh produce (Kim et al., 2009). However, consumption of raw sprouts has also been associated with outbreaks of foodborne illness (Dechet et al., 2014; Millan-Sango et al., 2017). According to the U.S. Food and Drug Administration (FDA), the majority of these outbreaks in North America (more than 30 since 1996) have been attributed to alfalfa sprouts (Gensheimer and Gubernot, 2016). This could be due to alfalfa sprouts being one of the more popular forms of seed sprouts since their consumption has increased in recent decades (Hong and Kang, 2016). The most common pathogens associated with consumption of raw sprouts are *Salmonella* and pathogenic *Escherichia coli* (DeWaal et al., 2000; Ding and Fu, 2016; Erdozain et al., 2013; Gensheimer and Gubernot, 2016; Landry et al., 2016). Since sprouts are often consumed raw in order to retain all their nutrition value, contaminated sprouts can easily be implicated in

foodborne illnesses (Millan-Sango et al., 2017). Potential sources for contaminating sprouts with pathogenic bacteria are various such as inadequately treated animal manure fertilizers, contaminated irrigation water, poor equipment sanitation, and mishandling by workers. Based on previous outbreaks and epidemiological investigations, seeds are the most likely source of sprout-associated outbreaks (NACMCF, 1999a). Pathogens trapped in or on seeds proliferate exponentially during sprouting and lead to foodborne illnesses (NACMCF, 1999b). The warm and humid environment provides favorable conditions for pathogens to multiply rapidly and thus presents a high risk of illness (Hong and Kang, 2016). In an attempt to reduce alfalfa sprout-related outbreaks, a number of decontamination studies such as gamma irradiation (Thayer et al., 2003), electrolyzed oxidizing water (Kim et al., 2003; Sharma and Demirci, 2003b), heat treatment (Bari et al., 2008; Feng et al., 2007; Neetoo et al., 2009; Suslow et al., 2002), sanitizers (Bari et al., 2003; Chang et al., 2010; Kumar et al., 2006; Weissinger and Beuchat, 2000; Zhao et al., 2010), UV irradiation (Sharma and Demirci, 2003a),

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and high hydrostatic pressure (Neetoo and Chen, 2010; Neetoo et al., 2008) have been conducted to develop intervention technologies. In particularly, non-thermal treatments represent attractive aspects for inactivation and controlling pathogens on seeds because these treatments do not compromise plant development and growth (Trinetta et al., 2011).

Among non-thermal technologies, ultraviolet (UV) irradiation is considered a promising non-thermal technology due to its ability to disinfect most types of microorganisms (Bintsis et al., 2000; Koutchma, 2009). Typically, UV disinfection is achieved by using either low-pressure (LP) or medium pressure (MP) mercury lamps (Tsenter et al., 2015). However, these lamps are constructed from fragile quartz material and contain toxic mercury, thus it can harmfully affect the environment (Chevremont et al., 2013; Close et al., 2006). As an alternative mercury-free source, there are dielectric barrier discharge (DBD)-driven excilamps (excimer or exciplex lamps) which are regarded as an attractive alternative to conventional mercury lamps due to wave-selective application, absence of mercury, fast-warming, and long life time (Matafonova and Batoev, 2012; Sosnin, 2007). These mercury-free lamps are based on the transition of rare gas-excited dimers, halogen-excited dimers or a rare gas excited complex, and emit nearly monochromatic radiation at wavelengths ranging from 172 to 345 nm depending on the type of rare gas and halogen used (Matafonova et al., 2013; Oppenländer, 2007; Sosnin et al., 2006). However, to our knowledge, there have been no studies evaluating the antimicrobial efficacy of this relatively new excilamp technology against foodborne pathogens on seed surfaces.

Accordingly, we examined the possibility of applying a Krypton-Chlorine (KrCl) excilamp as a seed sterilization intervention by evaluating its antimicrobial efficacy against *E. coli* O157:H7 and *S.* Typhimurium on alfalfa seed surfaces compared to a conventional LP mercury lamp.

2. Materials and methods

2.1. Bacterial strains and cell suspension

Three strains of *E. coli* (ATCC 35150, ATCC 43889 and ATCC 43890) and *S.* Typhimurium (ATCC 19585, ATCC 43971, and DT 104) were provided by the bacterial culture collection of the School of Food Science, Seoul National University (Seoul, South Korea), for this study. Stock cultures were prepared by growing strains in 5 ml of tryptic soy broth (TSB; Difco, BD) at 37 °C for 24 h, combining 0.7 ml with 0.3 ml of sterile 50% glycerol and then storing at −80 °C. Working cultures were streaked onto Tryptic Soy Agar (TSA; Difco, BD), incubated at 37 °C for 24 h and stored at 4 °C for less than 1 mo. All strains of *E. coli* O157:H7 and *S.* Typhimurium were cultured individually in 5 ml of tryptic soy broth (TSB; Difco, Sparks, MD, USA) at 37 °C for 24 h, followed by centrifugation ($4000 \times g$ for 20 min at 4 °C) and washed three times with sterile 0.2% peptone water (PW, Bacto, Sparks, MD). The final pellets were resuspended in 9 ml of sterile 0.2% PW to yield approximately 10^7–10^8 CFU/ml. The resuspended pellets of each strain of both pathogen species were combined to constitute a 2-pathogen mixed-culture cocktail.

2.2. Sample preparation and pathogen inoculation

Alfalfa seeds were purchased from a local seed retailer (Danong, Gyeonggi province, Korea Rep.). Fifty g of alfalfa seed were mixed with 9 ml of culture cocktail and gently agitated for 5 min. The pathogen-inoculated seeds were placed onto aluminum foil and dried in a laminar flow hood at 25 °C for 6 h to facilitate the attachment of bacteria before being subjected to UV treatments. In a preliminary experiment, the moisture content of inoculated seeds was measured during drying using a halogen moisture analyzer (HB43-S; Mettler Toledo, Columbus, OH) and compared to un-inoculated controls, and it was confirmed there

were no differences in moisture content after 6 h of drying (data not shown).

2.3. UV treatment

A DBD excilamp ($29 \times 9 \times 8$ cm; UNILAM, Ulsan, South Korea) filled with a KrCl gas mixture with output power of 20 W was used to decontaminate pathogens on seeds in this study. The excilamp was of cylindrical geometry covered by a metal case having an UV exit window with an area of 60 cm^2 (10 cm × 6 cm). A modulated electrical field was applied to a quartz glass body filled with KrCl gas. A conventional LP Hg lamp (G10T5/4P, 357 mm; Sankyo, Japan) with an output power of 16 W was used for comparison with the KrCl excilamp. The LP Hg lamp was inserted to an aluminum reflector to allow the irradiation to exit in one direction and the UV output window area was made of the same size as that of the KrCl excilamp (10 cm × 6 cm). These two lamp systems were placed vertically and directly above the samples and the distance between the emitters and sample was 13 cm. At that distance at the sample location, radiation intensities were measured with a UV fiber optic spectrometer (AvaSpec-ULS2048; Avantes, Eerbeek, Netherlands) and the light intensities of the KrCl excilamp and LP Hg lamp were 0.29 and 0.87 mW/cm^2, respectively.

Five g of inoculated alfalfa seeds were spread evenly into 90 mm diameter petri dishes and then treated at room temperature (25 ± 2 °C) with the 222 nm KrCl excilamp or 254 nm LP Hg lamp for 300, 600, and 900 s or 100, 200, and 300 s at equal dosages of 87, 174, and 261 mJ/cm^2; UV doses were calculated by multiplying irradiance values by the irradiation times.

2.4. Bacterial enumeration

For enumeration of pathogens, 5 g of treated samples were immediately transferred into sterile stomacher bags (Labplas Inc., Sainte-Julie, Quebec, Canada) containing 45 ml of sterile 0.2% PW. Stomacher bags containing treated samples were homogenized with a stomacher (EASY MIX, AES Chemunex, Rennes, France) for 2 min. After homogenization, 1 ml aliquots of stomached samples were tenfold serially diluted in 9 ml of sterile 0.2% buffered peptone water and 0.1 ml aliquots of samples or diluents were spread-plated onto selective media. Xylose lysine desoxycholate agar (XLD; Difco) and Sorbitol MacConkey Agar (SMAC; Difco) were used as selective media for the enumeration of *S.* Typhimurium and *E. coli*, respectively. All plates were incubated at 37 °C for 24 h before counting. Colonies were enumerated and calculated as $\log_{10}$ CFU/g.

2.5. Enumeration of injured cells

The over-lay method was used to count injured cells of *S.* Typhimurium (Lee and Kang, 2001). TSA, a nonselective medium, was used to resuscitate injured cells. One-tenth milliliter aliquots of appropriate dilutions were spread-plated in duplicate, and the plates were incubated at 37 °C for 2 h to enable injured cells to recover. The resuscitated media plates were then overlaid with 7 ml of the selective medium XLD for *S.* Typhimurium. The solidified plates were incubated at 37 °C for an additional 22 h. After incubation, typical black colonies characteristic of *S.* Typhimurium were counted. Enumeration of injured *E. coli* O157:H7 cells was accomplished with phenol red agar base (Difco) with 1% sorbitol (SPRAB) (Rhee et al., 2003). After incubation at 37 °C for 24 h, white colonies characteristic of *E. coli* O157:H7 were enumerated, and simultaneously, serological confirmation (RIM; *E. coli* O157:H7 latex agglutination test; Remel, Lenexa, KS, USA) was performed on randomly selected presumptive *E. coli* O157:H7 colonies.

2.6. Effect of KrCl excilamp and LP Hg lamp on viability of alfalfa seeds

Two hundred randomly selected treated or control seeds were

spread on sterile cheesecloth in 90 mm diameter petri dishes, and periodically provided with sufficient distilled water (DW) to foster seed germination. The seeds were incubated at room temperature ($25 \pm 2\,°C$) for 5 days. Only seeds with a hypocotyl protruding were counted as a sprout.

2.7. Seed DNA integrity assay

A mortar and pestle were pre-cooled with liquid nitrogen. After the liquid nitrogen was boiled off, five gram of untreated control seeds, or seeds treated with the 222 nm KrCl excilamp or 254 nm LP Hg lamp at a dosage of $261\,mJ/cm^2$ were placed in the mortar and additional liquid nitrogen was added. To completely dissociate the sample into a homogenous mixture for DNA extraction, seeds were ground by compressing the pestle against the mortar to tear or fragment the sample in the presence of liquid nitrogen (Mićić, 2016). DNA was extracted from ground seeds using a PowerPlant Pro DNA isolation Kit (MoBio Laboratories, Carlsbad, CA, USA), following the manufacturer's directions. SYBR green I (1:10,000 dilution; Molecular Probes, Eugene, OR, USA) was applied to extracted DNA solution at working concentration (1:1) for 15 min at 37 °C. A 200 µl aliquot of each sample was transferred to a 96-well fluorescence microplate and fluorescence was measured with a spectrofluorophotometer (Spectramax M2e; Molecular Devices, CA, USA) at excitation and emission wavelengths of 485 and 525 nm, respectively.

2.8. Statistical analysis

All experiments were repeated 3 times with duplicate samples. Data were analyzed by the ANOVA procedure of SAS (Version 9.4. SAS Institute Inc., Cary, NC, USA) and mean values were separated using the LSD *t*-test. Significant differences between values were determined at a probability level of $P < 0.05$.

3. Results and discussion

The primary purpose of this study was to investigate the applicability of the KrCl excilamp as a sterilization vehicle for alfalfa seeds. The bactericidal effect of the 222-nm KrCl excilamp and 254-nm LP Hg lamp against *S*. Typhimurium and *E. coli* O157:H7 on alfalfa seeds is presented in Table 1. Initial bacterial concentrations of *E. coli* O157:H7 and *S*. Typhimurium on alfalfa seeds were 6.20 and 6.39 log CFU/g, respectively. As irradiation dose of both lamps increased from 0 to $261\,mJ/cm^2$, the viable-count levels of both pathogens decreased. For the 222-nm KrCl excilamp and 254-nm LP Hg lamp at a dose of $261\,mJ/cm^2$, inactivation levels of *E. coli* O157:H7 were 2.77 and 1.43 log CFU/g, respectively, and those of *S*. typhimurium were 3.04 and 1.85 log CFU/g, respectively. These results reveal that higher irradiation doses produced higher inactivation efficacy and the 222-nm KrCl excilamp

Table 2
Alfalfa seed germination percentages after 254-nm LP Hg lamp or 222-nm KrCl excilamp treatment.

Treatment type	Treatment Dose (mJ/cm^2)	Germination rate (%)[a]
Untreated control	0	95.3 ± 0.58 A
LP Hg lamp (254-nm)	87	87.0 ± 1.00 C
	174	80.7 ± 2.08 D
	261	74.0 ± 2.65 E
KrCl excilamp (222-nm)	87	95.0 ± 1.00 A
	174	95.0 ± 0.00 A
	261	93.3 ± 0.58 B

Values in the same column followed by the same letter are not significantly different ($P > 0.05$).

[a] The values are means $\pm$ standard deviations from three replications.

yielded significantly ($P < 0.05$) greater bactericidal capacity for both pathogens than did the 254-nm LP Hg lamp. Moreover, Table 1 also shows that the 222-nm KrCl excilamp and 254-nm LP Hg lamp did not produce significant ($P > 0.05$) differences between reduction levels enumerated on selective agar and those on recovery agar, indicating that generation of sublethally injured cells was not significant for either pathogen. In general, it is known that the 222-nm KrCl excilamp demonstrates more bactericidal efficacy than the 254-nm LP Hg lamp based on several previous studies (Ha et al., 2017; Kang et al., 2018; Wang et al., 2010; Yin et al., 2015) and this tendency was also apparent in the present study for the reduction of pathogens on alfalfa seed surfaces. The greater efficacy of the 222-nm KrCl excilamp is known to be due to multi-target damage due to absorption by various components (proteins, lipids, and others) in the cell and ROS generation when exposed to wavelengths in this bandwidth; conversely, reduction by 254-nm LP Hg lamps is mostly due to DNA damage (Ha et al., 2017; Kang et al., 2018).

In order to be utilized by the sprouts industry, this 222-nm KrCl excilamp disinfection system should be able to achieve commercially acceptable sprout yield by not affecting seed viability. So, we determined seed viability after each UV treatment by measuring the germination rate. In the untreated control the germination rate was 95%. As shown in Table 2, when the 222-nm KrCl excilamp was applied at 87, 174, and $261\,mJ/cm^2$, the germination rates were 95, 95, and 93%, respectively. When the 254-nm LP Hg lamp was applied at 87, 174, and $261\,mJ/cm^2$, the germination rates were 87, 81, and 74%, respectively. In other words, the 222-nm KrCl excilamp showed a significantly lower ($P < 0.05$) germination rate than the untreated control at $261\,mJ/cm^2$, but still maintained higher than 90% germination, whereas the 254-nm LP lamp showed a significantly ($P < 0.05$) lower value than the untreated control at only $87\,mJ/cm^2$ and this value decreased with increasing treatment dose such that the germination rate was less than 80% at $261\,mJ/cm^2$. Therefore, it can be seen that the KrCl excilamp is more suitable as a seed sterilization system than the LP Hg lamp.

Table 1
Log reductions of *E. coli* O157:H7 and *S*. Typhimurium on culture media treated with a 254-nm LP Hg lamp or 222-nm KrCl excilamp.

Treatment type	Dose (mJ/cm^2)	Log reduction [$\log_{10}$ (N_0/N)][a] by organism and selection medium			
		E. coli O157:H7		*S*. Typhimurium	
		SMAC	SPRAB	XLD	OV-XLD
254-nm LP Hg lamp	87	0.70 ± 0.06Aa	0.71 ± 0.54Aa	0.75 ± 0.21Aa	0.43 ± 0.24Aa
	174	1.16 ± 0.32ABa	1.39 ± 0.10ABa	1.15 ± 0.38ABa	1.01 ± 0.61Aa
	261	1.43 ± 0.39Ba	1.87 ± 0.48Ba	1.85 ± 0.23Ba	1.42 ± 0.55Aa
222-nm KrCl excilamp	87	0.85 ± 0.45Aa	1.36 ± 0.11Aa	1.22 ± 0.20Aa	1.31 ± 0.37Aa
	174	1.77 ± 0.10Ba	1.83 ± 0.31Ba	2.27 ± 0.51Ba	2.06 ± 0.65ABa
	261	2.77 ± 0.10Ca	2.97 ± 0.07Ca	3.04 ± 0.54Ca	2.64 ± 0.12Ba

SMAC, sorbitol MacConkey agar; SPRAB, phenol red agar base with 1% sorbitol; XLD, xylose lysine desoxycholate agar; OV-XLD, overlay XLD agar on TSA.

[a] Values are means $\pm$ standard deviations from three replications. Values in the same column and treatment type followed by the same capital letter are not significantly different ($P > 0.05$). Values with the same lowercase letter in the same row are not significantly different ($P > 0.05$).

In this regard, we sought to find the reason why the germination rate was reduced after 254-nm LP Hg lamp treatment, while 222-nm KrCl treatment maintained a high germination rate. In general, DNA is known to be one of the major targets of UV-induced damage in both eukaryotes and prokaryotes (Nawkar et al., 2013). UV irradiation induces several types of DNA damage, such as generation of cyclobutyl pyrimidine dimers (CPDs), (6–4) photoproducts ((6–4) PPs) (Hutchinson, 1987), DNA double-strand breaks and Inter/Intra Cross Linkages (ICL) (Molinier et al., 2008). In order for seed germination to occur, a complex, multi-step process is initiated, which is accomplished through the coordinated expression of numerous genes in different tissues (Potokina et al., 2002). Indeed, Jiang et al. (2011) concluded that the occurrence of DNA damage after UV exposure can cause plant growth to be inhibited (Jiang et al., 2011). Thus, the difference in the germination rate of alfalfa seed may be considered to be related to differences in DNA damage induced between different UV sources (Excilamp and LP Hg lamp). Previous studies on the action of 222-nm KrCl excilamps and 254-nm LP Hg lamps have shown that DNA damage to pathogenic bacteria mainly occurs at the 254-nm wavelength region, whereas in the 222-nm wavelength region, components such as enzymes and cell membranes are primarily damaged (Kang et al., 2018). The 222-nm KrCl excilamp and 254-nm LP Hg lamp each induce damage to different cell components because of the difference in absorbance of UV radiation depending on the type of cell component. Specifically, the UV radiation absorption spectrum of DNA exhibits maximum absorption at 260 nm (Pattison and Davies, 2006; Taylor, 1994), while proteins have an additional higher absorption at between 180 and 230 nm (Clauss et al., 2009; Kerwin and Remmele Jr, 2007) and lipid components show increasing absorption from 250 nm to a peak centered at 198 and 209 nm (Spector et al., 1996). Therefore, we thought that a 254-nm LP Hg lamp, operating on the same principle as in bacteria, would have a negative effect on germination of alfalfa seed by inducing seed DNA damage due to high absorbance of seed DNA at 254 nm, thus, we measured the degree of DNA damage after each UV treatment by using SYBR green I. DNA damage was assessed using the property of abrupt increase in brightness while SYBR green I interacts with double-stranded DNA (dsDNA) (Dragan et al., 2012). In other words, when damage to DNA occurs, the fluorescence signal of SYBR green I decreases. Through this, the degree of DNA damage is evaluated. The results are shown in Fig. 1. The 222-nm KrCl excilamp treatment showed no significant difference

($P > 0.05$) in fluorescence signal compared to the untreated control but was significantly reduced ($P < 0.05$) when treated with the 254-nm LP Hg lamp. These results show that treatment with 254-nm LP Hg lamps can damage the DNA of seeds and adversely affect germination. Furthermore, since SYBR green I has the characteristic of interacting with double-stranded DNA, it can be deduced that DNA double-strand breakage is one of the causes of seed DNA damage induced by 254-nm LP Hg lamp treatment.

The results of this study demonstrate that the 222-nm KrCl excilamp inactivates *E. coli* O157: H7 and *S*. Typhimurium on alfalfa seed surfaces more efficiently than the 254-nm LP Hg lamp. It was also found that treatment with 254-nm LP Hg lamp had a detrimental effect on the germination rate of alfalfa seed, whereas 222-nm KrCl excilamp maintained high germinability. This is because the 222-nm KrCl excilamp did not affect the DNA of the seed, resulting in a higher germination rate, whereas the 254-nm LP Hg lamp caused damage to seed DNA and lowered the germination rate. However, since 222-nm KrCl excilamp treatment alone reduced *E. coli* O157: H7 and *S. typhimurium* by only 2–3 log, this intervention by itself cannot meet the 5-log reduction goal for foodborne pathogens on seed recommended by the National Advisory Committee on Microbiological Criteria for Foods (NACMCF) (NACMCF, 1999b). Furthermore, Sharma and Demirci (2003a) reported that as the thickness of the alfalfa seed layer increased, the decontamination effect of UV treatment on *E. coli* O157:H7 on alfalfa seed was reduced, which was attributed to its low permeability to UV light. Thus, it is expected that the decontamination effect of the 222-nm KrCl excilamp would decrease with increasing seed volume. Additionally, in this study, alfalfa seed surfaces were not evenly exposed to UV light because they were uniformly laid on a monolayer and subjected to UV treatment in a static state. This is because we have not been able to properly rotate alfalfa seeds in our lab. Therefore, for practical application by the seed industry, it is necessary to develop seed rotating technology that optimizes the disinfection effect of the 222-nm KrCl excilamp applied to a large-volume process and induces more effective treatment by allowing the seed to be uniformly exposed to UV light as a whole. Furthermore, we believe that it is essential to apply a hurdle approach that merges KrCl excilamp treatment with other disinfection technologies as well as applying rotational technology to meet or exceed this 5-log goal in the seed industry. Based on
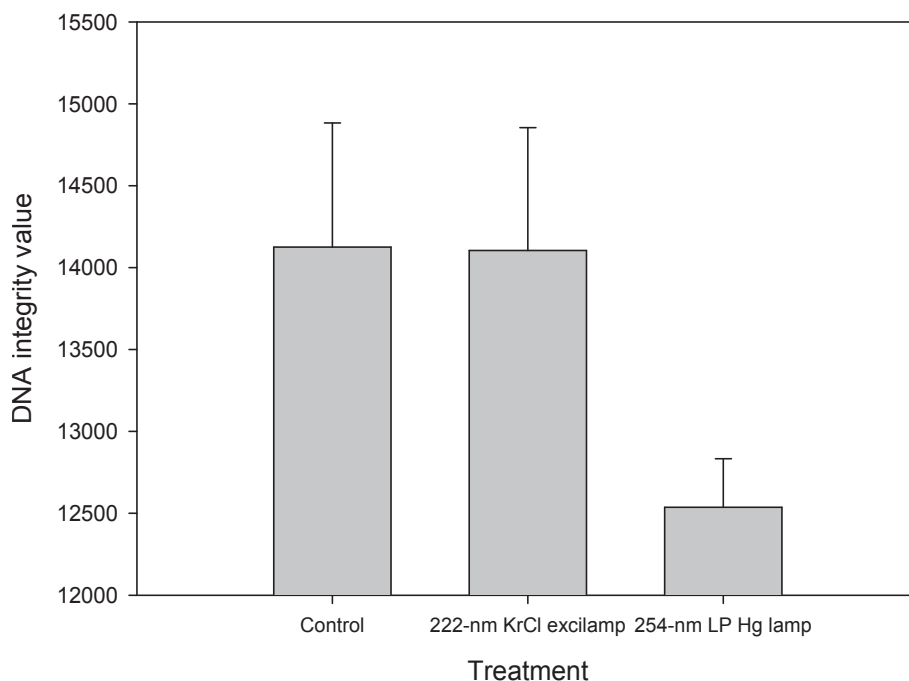

Fig. 1. Values of DNA integrity in alfalfa seed obtained from the DNA integrity assay using SYBR green I after 222-nm KrCl excilamp or 254-nm LP Hg lamp treatment at 261 mJ/cm^2.

this study, therefore, 222-nm KrCl excilamps could be used as a disinfection system by the seed industry in conjunction with another rotation and disinfection technology to give an additive or even synergistic effect to ensure safety of seed sprouts to reduce future episodes of sprout-borne illnesses. This is an objective requiring additional research.

Acknowledgments

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