Citrus R&T (e-ISSN 2236-3122)
Citrus Research & Technology, v. 38, n. 2, 2017
http://dx.doi.org/10.4322/crt.ICC098

Artigo/Article

Preliminary investigation of alkaline and acidic electrolysed water to control *Penicillium* species of citrus

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SUMMARY

Penicillium species can cause massive losses for high value crop like citrus. Electrolysed water was proposed as alternative treatment to chlorine compounds and fungicides. The effect of incubating spore suspension of three *Penicillium* spp. (*P. digitatum*, *P. italicum*, *P. ulaiense*) with acidic (acEW) and alkaline (alEW) electrolysed water was assessed. The effect of those treatments against artificially inoculated orange fruit was also investigated. The electrolysed water was produced by PE-1 water ionizer machine that use only naturally present salts in tap water and allows the user to choose between different levels of electrolysing. Five titanium-platinum coated plates were used for the electrolysing process, and the system had separation barrier between anode and cathode compartments to produce two types of electrolysed water. acEW and alEW physical/chemical characteristics were measured and studied in relation to inactivation of three *Penicillium* spp. units. Results revealed weak correlation between free chlorine, Oxidation/Reduction Potential (ORP) and pH against the strength of sanitization tested as colony forming units when using alEW. While free chlorine in acEW has obvious correlation with strength of biocidal effect, followed by ORP then pH. The higher levels in acEW and alEW showed the strongest biocidal effect on *Penicillium* spp. When using the optimum level of electrolysing, acEW achieved higher inhibition for *P. digitatum*, *P. italicum* and *P. ulaiense*. The percentage of reduction of decay incidence due to alEW was 20, 20 and 27% for green, blue and whisker moulds, respectively. In case of acEW, the percentage of reduction was 27, 33 and 33% for green, blue and whisker moulds, respectively as compared to water control. The results support the usefulness of electrolysed water in pathogen inactivation, but further experiments using amended salt solutions are needed to improve the biocidal effect of electrolysing.

Index terms: electrolysed water, green mould, blue mould and whisker mould.

Avaliação preliminar do uso de água eletrolisada acidificada e alcalina para o controle de espécies *Penicillium* em citros

RESUMO

As espécies de *Penicillium* podem causar perdas maciças para culturas de alto valor como citros. A água eletrolisada foi proposta como tratamento alternativo para compostos de cloro e fungicidas. O efeito da incubação da suspensão de esporos de três espécies de *Penicillium* spp. (*P. digitatum*,

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P. italicum, *P. ulaiense*) com água eletrolisada acidificada (AEAc) e alcalina (AEAl). O efeito desses tratamentos contra frutos de laranja inoculados artificialmente também foi investigado. A água eletrolisada foi produzida por uma máquina ionizadora de água PE-1 que usa apenas sais presentes naturalmente na água da torneira e permite ao usuário escolher entre diferentes níveis eletrolíticos. Foram utilizadas cinco placas revestidas com titânio e platina para o processo de eletrólise, e o sistema apresenta barreira de separação entre compartimentos de ânodo e cátodo para produzir dois tipos de água eletrolisada. As características físicas/químicas de AEAc e AEAl foram medidas e estudadas em relação à inativação unidades reprodutivas de três espécies de *Penicillium* spp.. Os resultados revelaram uma fraca correlação entre o cloro livre, o Potencial de Óxido-Redução (POR) e o pH contra a resistência à sanitização testada sobre a unidades formadoras de colônias ao usar AEAl. Enaqunato que o cloro livre em AEAc mostrou uma correlação óbvia com a força do efeito biocida, seguido de ORP e pH. Os níveis mais altos de AEAc e AEAl mostraram o efeito biocida mais forte em *Penicillium* spp. Ao usar o nível ótimo de eletrólise, o AEAc obteve uma inibição maior para *P. digitatum*, *P. italicum* e *P. ulaiense*. A porcentagem de redução da incidência de podridão com o uso de AEAl foi de 20, 20 e 27% para os bolores verde, azul e bigode, respectivamente. No caso de AEAc, a porcentagem de redução de incidência da doença foi de 27, 33 e 33% para os bolores verde, azul e bigode, respectivamente, em comparação com o controle (só com água). Os resultados mostram o potencial da utilização da água eletrolisada para inativação do fitopatógeno, porém, outros ensaios, utilizando soluções de sais modificadas, são necessárias para melhorar o efeito biocida da eletrólise.

Termos de indexação: água eletrolisada, bolor verde, bolor azul e bolor de aspecto bigode.

INTRODUCTION

Citrus green and blue moulds caused by *Penicillium digitatum* (Pers.:Fr.) Sacc., and *P. italicum* Wehmer, respectively, are the most serious postharvest diseases causing significant losses (Holmes & Eckert, 1999; Zhu et al., 2006). *P. ulaiense* Hsieh, Su & Tzeancan cause a citrus postharvest rot from natural infection in Egypt (Youssef et al., 2010). Development of alternative methods for sanitizing fresh products and controlling postharvest diseases are derived by economic and consumer demand motivations (Mahajan et al., 2014).

Electrolysed water has also been classified as functional water and some scientists have used the terminology of acidic electrolysed water (AcEW or AEW) instead of electrolysed water. Similarly, alkaline electrolysed water (AlEW) has also been referred to as electrolysed reducing (ER) water or basic electrolysed water (BEW) (Al-Haq et al., 2005). Electrolysed water (EW) was introduced as decontaminating agent (Shimizu & Hurusawa, 1992), and its application was approved in Japan and USA as food additive and sanitizing agent (Park et al., 2002; Yoshida et al., 2004). EW has shown its potential to inactivate pathogenic microorganisms such as bacteria (Kim et al., 2000a; Fabrizio & Cutter, 2003; Vorobjeva et al., 2004) and fungi (Al-Haq et al., 2001, 2002; Buck et al., 2003; Okull & Laborde, 2004). One of the main advantages of electrolysed water its less adverse effect on environment and human health because no hazardous materials are used during its production (Park et al., 2002).

Salts such as KCl and $MgCl_2$ have been used for electrolyzing (Buck et al., 2002), while NaCl is the salt used for food processing applications. Since tap water contains chloride, it is also possible to reactivate free chlorine in tap water by electrolysis (Nakajima et al., 2004). To the best of our knowledge, no previous studies were carried out to investigate the effect of electrolysing using naturally available salts present in tap water on *Penicillium* spp. attacking citrus fruit. PE-1 water ionizer machine which electrolysis the tap water utilizing the naturally available salts, without the need to amend any further additives and also produces both acidic and alkaline electrolysed water.

Our hypothesis is to use the alkaline and acidic electrolysed water generated by natural salts already present in tap water without adding any other salts to inhibit the pathogenic units of *Penicillium* spp. causing fruit decay in citrus. The current study was carried out to investigate the effect of acidic and alkaline electrolysed water, produced using natural salts already present in tap water, against *P. digitatum*, *P. italicum* and *P. ulaiense* *in vitro* and *in vivo* under artificial inoculation.

MATERIAL AND METHODS

Fungal preparation

Three species of Pencillium were used in this study. The isolates of *P. digitatum*, *P. italicum* and *P. ulaiense* were obtained from symptomatic orange fruits cv. Valencia late. Each culture was grown individually on PDA plates for 1 week at 24 °C. Spore suspension was prepared by flooding the cultures with sterile distilled water containing 0.05% Tween 80 (v/v), and then conidia were dislodged by scraping the surface with a sterile spatula. The resulting suspension was filtered through two layers of sterile cheesecloth. The spore counts were made by a haemocytometer (Thoma- Tiefe 0.100 mm, 1/400 qmm, Lutzellinden, Germany) and the suspension was adjusted with sterile distilled water to obtain a final concentration of 10^5 ml^{-1} conidia.

Preparation and treatment of electrolysed water

In these experiments, electrolysed water was obtained by PE-1 model and two-factor composite experimental design was adopted to investigate the effect of time of electrolysis (30, 60 and 120 min) and type of water produced; alkaline (with three levels; 1, 2 and 3) and acidic (with two levels; 1 and 2), on electrolysis efficiency tested on spore suspension of the 3 *Penicillium* spp. For the electrolysis process, five titanium-platinum coated plates are used to produce electrolysed water with pH levels vary from 3 to 11 and ORP level vary from +500mv to -800mv.

Assessment of electrolysed water as sanitizing agent on spore suspension of three *Penicillium* spp.

The effect of the above electrolysed water types on pathogen inactivation (*P. digitatum*, *P. italicum* and *P. ulaiense*) was studied. Volumes of 100 µl of each *Penicillium* species suspension with estimated concentration of 10^5 conidia ml^{-1} as prepared above were added to the produced electrolysed water and incubated for 10 min at room temperature. In order to keep the number of colonies expected to grow on the 9 cm Petri dishes at a countable limit, a 10-fold dilution was made for each treatment by mixing 100 µl of the conidial suspension + electrolysed water with 900 µl of sterilized distilled water. Volumes of 100 µl from the mixture were spread on PDA amended with 250 mg L^{-1} of ampicillin and 250 mg L^{-1} of streptomycin on 9 cm Petri dishes. The PDA plates were incubated at 24 °C and colony forming units (CFUs) were recorded after 3-4 days. Filtered and distilled water were used as controls and each treatment contains five PDA plates as replicates.

Properties of electrolysed water and its correlation to the efficacy

The properties of the electrolysed water produced with each parameter(s) were measured as; pH and oxidation/reduction potential (ORP) (pH meter- Adwa- AD8000) and free chlorine using (Lovibond photometer- MD100).

Assessment of the effect of alkaline and acidic electrolysed water on Penicillium decay *in vivo*

Based on the *in vitro* results, the best parameters for both alkaline (level 3-60 min) and acidic water (level 2-120 min) were tested *in vivo* under artificial inoculation. Oranges fruit cv. Valencia late were collected after maturity and washed excessively with water and soap. The surface sterilisation was performed by treating fruits with NaOCl solution (1%) and then rinsed with sterilised water. In each fruit, one wound (5 mm wide x 3 mm deep) was made at the equatorial region using sterile needle. For each treatment, 20-30 µl of alkaline or acidic electrolysed water was applied in each wound. Under the same conditions, distilled water was served as control. After two hours at room temperature, 20 µl of the pathogen conidial suspension was applied in the same wound. A completely randomized block experiment was designed and carried out. Each treatment consisted of three replicates and each replicate consisted of 5 fruits. The fruits were incubated at 24 °C and 90-95% RH for two weeks. Rot incidence (%) and lesion diameter (mm) were measured.

Statistical analysis

Percentage data were arcsine-square root transformed to normalize variance before ANOVA analysis. Mean values of treatments were compared using Fisher's protected Least Significant Difference (LSD) and judged at $p \leq 0.05$ level. When applicable, regression analysis was performed using Excel.

RESULTS

Properties of electrolysed water and its correlation to the efficacy

The analysis for the parameters of alkaline water produced by PE-1 machine and their correlation to recovered pathogen count has shown a general insignificant correlation (Figure 1, Figure 2 and Figure 3). Although a tendency toward gradient effect of ORP and pH can be seen on CFU of pathogen cells, but probably due to weak electrolysing capacity of PE-1 machine (usually for lab-based and household uses) and short exposure time. On the contrary, the acidic water produced by PE-1 machine has shown higher correlation between water parameters and recovered pathogen count. Free chlorine has shown the strongest correlation (Figure 4) followed by ORP then pH (Figure 5 and Figure 6). As a general comparison, effect of acidic water produced by PE-1 machine was more evident in reducing viability of pathogen unites compared to alkaline water, which allowed for better estimation of the correlation between water parameters and pathogen count.

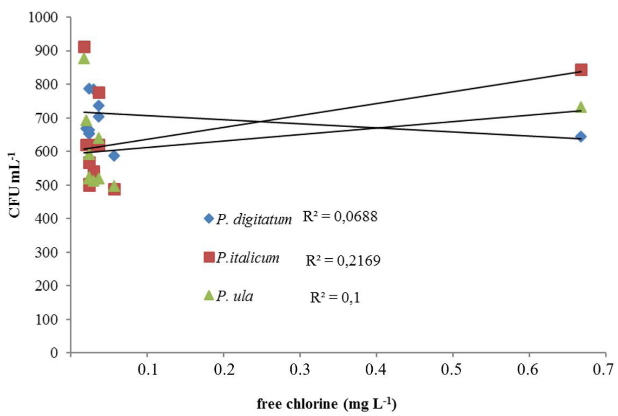

Figure 1. Correlation between free chlorine of alkaline water produced by PE-1 machine and count of 3 pathogen species; *P. digitatum, P. italicum* and *P. ulaiense*. Regression coefficient represented by R^2 value.

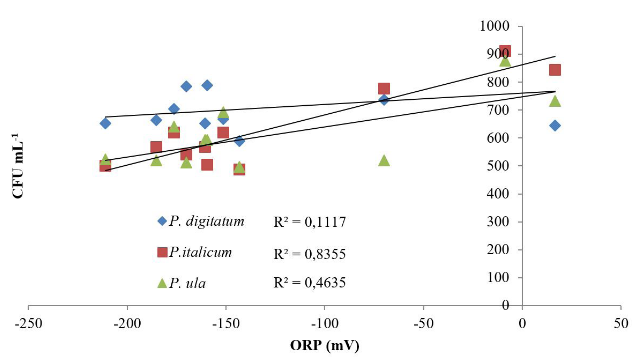

Figure 2. Correlation between ORP of alkaline water produced by PE-1 machine and count of 3 pathogen species; *P. digitatum, P. italicum* and *P. ulaiense*. Regression coefficient represented by R^2 value.

Assessment of electrolysed water as sanitizing agent on spore suspension of three *Penicillium* spp.

For the PE-1 machine parameters, all adjustments have shown significant biocidal effect on the three *Penicillium* spp

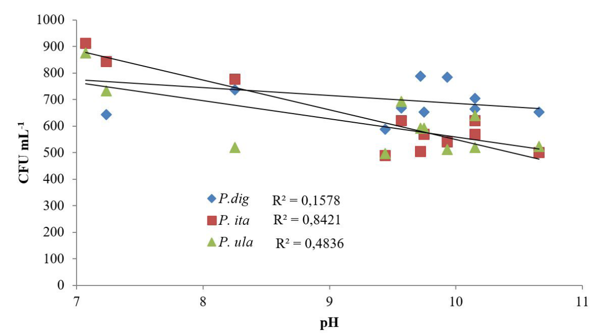

Figure 3. Correlation between pH of alkaline water produced by PE-1 machine and count of the 3 pathogen species; *P. digitatum, P. italicum* and *P. ulaiense.* Regression coefficient represented by R^2 value.

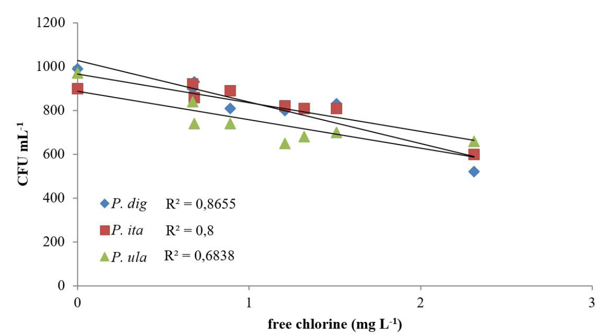

Figure 4. Correlation between free chlorine of acidic water produced by PE-1 machine and count of 3 pathogen species; *P. digitatum, P. italicum* and *P. ulaiense*. Regression coefficient represented by R^2 value.

compared to distilled water that was used as negative control. Meanwhile, filtered water (non-electrolysed water) has shown significant effect on pathogen count compared to distilled water, which may be attributed mainly to chlorine added in water treatment plants. Variability in concentration of chlorine added in water treatment plants makes the correction for its effect a problematic issue. The strongest effect on *P. digitatum* was achieved by level 3 at 60 min electrolysing time. Other machine parameters that have achieved statistically similar effect were: all electrolysing timing at level 1, and level 3 at timing 120 min. While electrolysing at level 2 all timing have lower effect on *P. digitatum* population. For the effect of machine parameters on *P. italicum*, electrolysing at level 3 timing 60 min has shown the strongest biocidal effect, followed by group that have statistically similar effect; level 1-60 min, level 2-30 min, level 3-30 min. Then level 1-120 min and level 3-120 min. Similarly, for *P. ulaiense*, electrolysing at level 3 timing 60 min has shown the most effective inactivation of pathogen. Also, level 1-60 min, level 1-120 min, level 2-30 min, level 2-60 min, level 3-30 min, and level 3-120 min have shown statistically similar effect. Generally, *P. ulaiense* has shown the most sensitivity toward electrolysing water, followed by *P. italicum* and then *P. digitatum*. And generally, electrolysing at level 3 timing 60 min has shown the most evident effect on all pathogens (Figure 7).

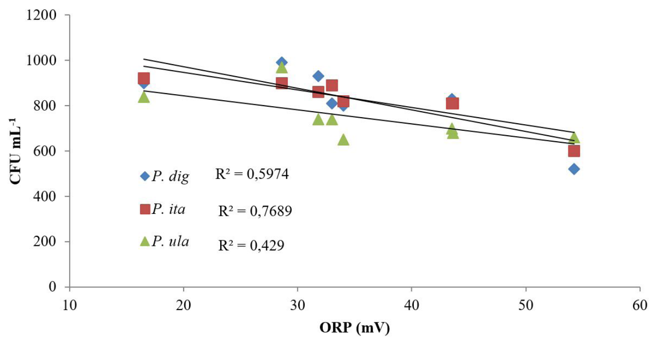

Figure 5. Correlation between ORP of acidic water produced by PE-1 machine and count of 3 pathogen species; *P. digitatum, P. italicum* and *P. ulaiense.* Regression coefficient represented by R^2 value.

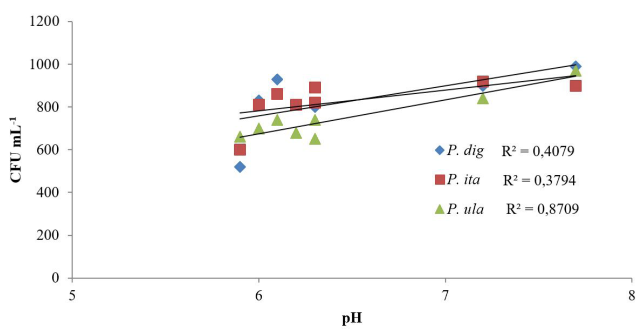

Figure 6. Correlation between pH of acidic water produced by PE-1 machine and count of 3 pathogen species; *P. digitatum*, *P. italicum* and *P. ulaiense*. Regression coefficient represented by R^2 value.

For acidic water, the machine allows only two levels, and 3 timing that tested 30, 60, 120 min. The acidic water for all levels and timing has shown significant effect compared to distilled water and filtered water. The acidic water contains higher levels of chlorine, which was reflected on the stronger effect of acidic water, even though compared to filtered water that contained chlorine added in water treatment plants. The most apparent effect was shown by electrolysing water at level 2-120 min, while electrolysing at level 2-30 min, level 2-60 min, level 1-120 min have shown comparable effects. The effect of electrolysed acidic water has shown clearly its robust effect on *P. ulaiense*, followed by *P. italicum* then *P. digitatum* (Figure 8).

Assessment of the effect of alkaline and acidic electrolysed water on *Penicillium* decay *in vivo*

The percentage of reduction of decay incidence due to alkaline electrolysed (level 3-60 min) water was 20, 20 and 27% for green, blue and whisker moulds, respectively. In case of acidic electrolysed water (level 2-120 min), the percentage of reduction was 27, 33 and 33% for green, blue and whisker moulds, respectively as compared to water control (Figure 9). Concerning lesion diameter, no significant difference was observed between treatments (Figure 10). Comparing the results obtained from the two parameters measured; disease incidence and lesion diameter has highlighted that the apparent effect is on disease incidence rather than strength of the lesion diameter.

DISCUSSION

The aim of current study was to investigate the effect of acidic and alkaline electrolysed water, produced using natural salts already present in tap water, against *P. digitatum*, *P. italicum* and *P. ulaiense in vitro* and *in vivo* under artificial inoculation.

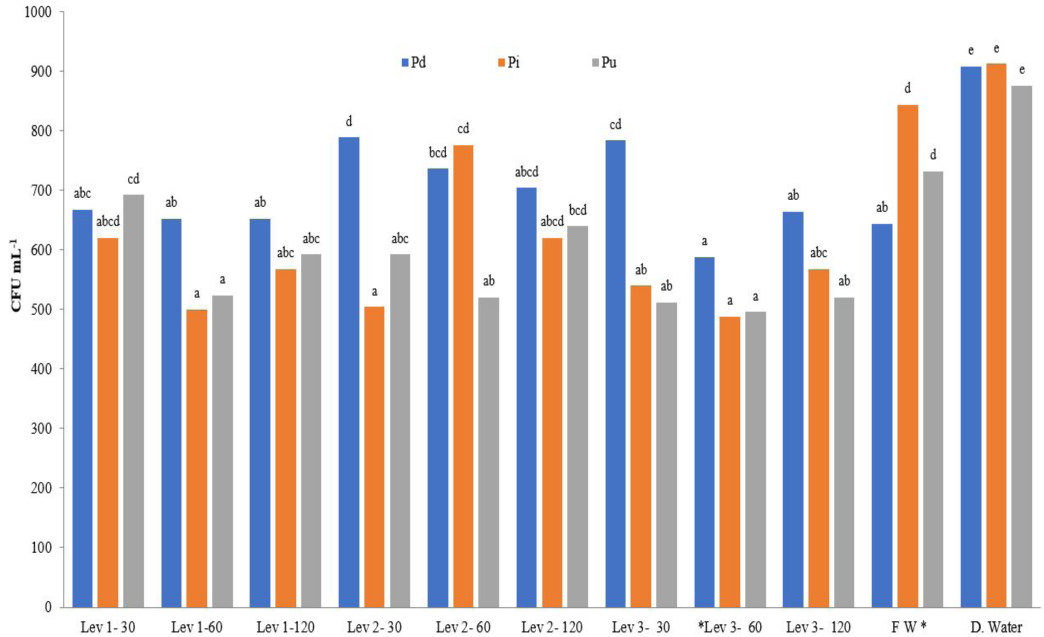

Figure 7. Effect of alkaline water produced by PE-1 machine different parameters on *P. digitatum, P. italicum* and *P. ulaiense* CFU count. Bars with similar litters indicate non-significant difference according to Fisher's protected Least Significant Difference (LSD) at $p \leq 0.05$. Post hoc analysis was performed between different levels and time and within the same pathogen.

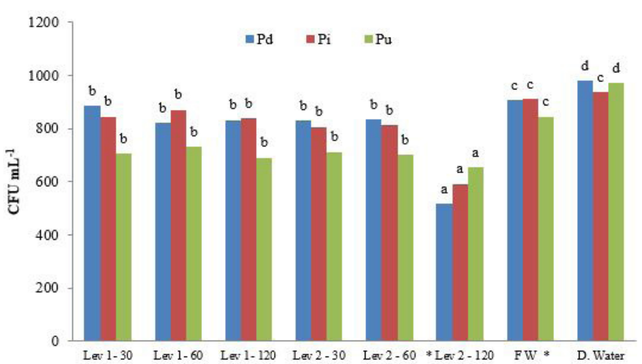

Figure 8. Effect of acidic water produced by PE-1 machine different parameters on *P. digitatum, P. italicum* and *P. ulaiense* CFU count. Bars with similar litters indicate non-significant difference according to Fisher's protected Least Significant Difference (LSD) at $p \leq 0.05$. Post hoc analysis was performed between different levels and time and within the same pathogen.

The effect of chlorine as a strong sanitizer is representing the milestone in the biocidal effect of electrolysed water as has been suggested by many researchers (Kim et al., 2000a,b; Park et al., 2004). The Cl- free anions generated from electrical dissociation of Cl- salts, the augmented NaCl salt are strong oxidizers that interfere with multiple biological structures and functions (Koseki & Itoh, 2000; Kiura et al., 2002; Mahmoud et al., 2004). Anions of Cl-are targeting polypeptides and carbohydrates in the cell wall, in addition to destruction of nucleic acids – DNA and RNA – which impairs the process of DNA replication and gene expression, therefore, halt the cell division and also stop the essential biological function leading to cell death.

Oxidation/Reduction Potential (ORP) has shown moderate biocidal effect following free chlorine level. ORP is representing the strength of ions to accept/donate electrons (Reduction/Oxidation), and was suggested as alternative more strong sanitizing factor compared to free chlorine (Venkitanarayanan et al., 1999; Kim et al., 2000b; Al-Haq et al., 2002; Liao et al., 2007). Kim et al. 2000 findings supports that in case of long exposure time, decrease of ORP to -300 mV will reduce coliform bacteria population 10 fold, at ORP -400 will coliform bacteria population will decrease 100 fold, and decreasing ORP to below -600 will completely inactivate all coliform bacteria. In this study, ORP is representing the second more important sanitizing agent after free chlorine. Suggestions of Kim et al. (2000b) that biocidal activity of electolysed water can be improved by manipulating

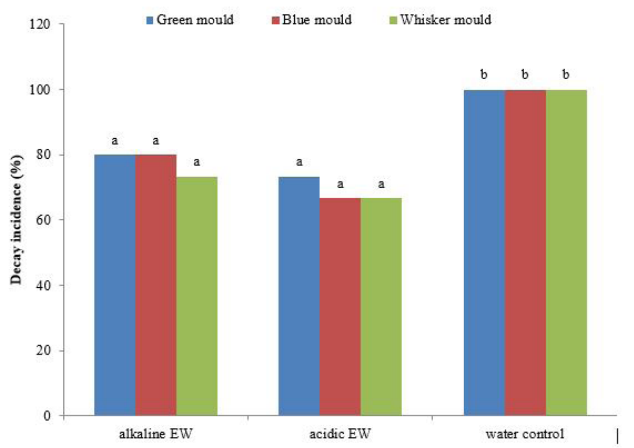

Figure 9. Effect of alkaline and acidic electrolysed water, *in vivo*, against green, blue and whisker moulds assessed by disease incidence (DI) in Valencia late oranges artificially inoculated with the spore suspension. Bars with similar litters indicate non-significant difference according to Fisher's protected Least Significant Difference (LSD) at $p \leq 0.05$.

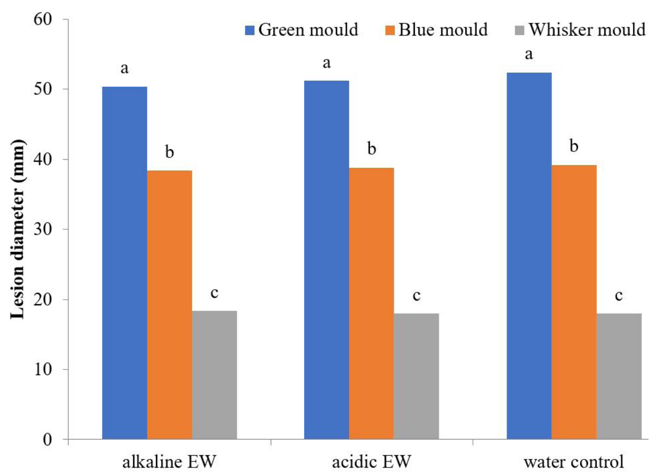

Figure 10. Effect of alkaline and acidic electrolysed water, *in vivo*, against green, blue and whisker moulds assessed by lesion diameter (LD) in Valencia late oranges artificially inoculated with the spore suspension. Bars with similar litters indicate non-significant difference according to Fisher's protected Least Significant Difference (LSD) at $p \leq 0.05$.

the ORP regardless the anion present, which propose that using salts with different anion radical than Cl- can still achieve the same level of disinfestation or can even improve the biocidal activity targeting multiple sites in the pathogen biological system.

The values of pH represent the level of hydrogen ion activity governing the solution acidity/alkalinity. This study shows that solution acidity/alkalinity has a biocidal effect against pathogen cells, and although came third to free chlorine and ORP, but still show significant effect on pathogen count. The role of pH is believed to make the cells more sensitive to active chlorine by erupting their outer membrane and facilitate the entry of HOCl (Park et al., 2004).

The PE-1 machine using the higher level – either level 2 or level 3 in acidic and alkaline water respectively, are showing the strongest biocidal effect on the three *Penicillium* spp. While variability can be seen for the effect of electrolysing time, using electrolysing time 60 min or more may achieve improved effect. Meanwhile, acidic water has shown higher biocidal activity toward three *Penicillium* spp when compared to the effect of alkaline water. And *P. ulaiense* has shown the most sensitivity towards electrolysed water, followed by *P. italicum* and then *P. digitatum*. Previous studies have suggested the higher biocidal effect of acidic water, and suggested alkaline water to be used in cleaning and degreasing before application of acidic electrolysed water (Koseki & Itoh, 2000; Fabrizio et al., 2002; Koseki et al., 2004; Ayebah et al., 2005; Bosilevac et al., 2005). The low percentage of reduction obtained from *in vivo* experiments may be due to the low quantity of natural salts already present in tap water. As a conclusion, the results support the usefulness of electrolysed water in pathogen inactivation, but further experiments using amended salt solutions are needed to improve the biocidal effect of electrolysing.

ACKNOWLEDGEMENTS

Current work was supported by the Science and Technology Development Fund (STDF), Egypt (grant no. 5555 Basic & Applied).

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Received: September 12, 2017
Accepted: December 17, 2017