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Electric pulses decrease the growth activity of *Erwinia amylovora* bacterium

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ABSTRACT

The current work studies the exposure effect of Erwinia *amylovora* to extremely low-frequency square positive electric pulses (ELF-EP) at constant field strength of 400V/m at 28°C for various frequencies in the range 0.7-1.6 Hz. The results show that *E. amylovora* exposed to 1.4 Hz differs from unexposed in growth characteristic curve. The results were confirmed through DNA analysis which indicated that the change occurs only in the bacterial cell wall. Also, the results from Enterobacterial repetitive intergenic consensus (Eric-PCR), Box-PCR, Repetitive extragenic palindromic (Rep-PCR) indicated that there was no difference between unexposed and exposed bacteria to 1.4 Hz ELF-EP for different exposure durations. Therefore, it concluded that electric pulsed fields for 1.4 Hz can be used to control the growth activities of *E. amylovora.*

Keywords: ELF-EP, *Erwinia amylovora*, PCR

INTRODUCTION

E. amylovora is a gram-negative bacterium. It is considered the main cause of fire blight disease on the pear tree (Rosselo *et al*., 2006). Infected plant parts will, in all cases cause a sticky, amber-like, drop of ooze, water-soaked, turn dark green, wilt, and finally turn brownish to black (Vrancken *et al*., 2013). According to the European and Mediterranean Plant Protection Organization (EPPO, 2012), *E. amylovora* is present in more than 50 countries around the world, depending on the occurrence of favourable climatic conditions, the amount of initial inoculum and pathogen virulence, and the susceptibility of host species (Rosselo *et al*., 2006). In Egypt, the serious symptoms of this disease were mostly a blight lesion and caused the loss of 10-75% of tree flowers/trees (Parthasarathy *et al*., 2021). Streptomycin and Oxytetracycline can control fire blight effectively, but they have a probability of producing resistance of pathogenic strains. However, chemical treatments like copper fungicides can restrain the effective development of the disease (Paulin and Lachaud, 1984). However, the chemical treatments have limited success due to their toxicity and their effect on human and their pollution of the environment. Researchers are seeking to discover new environmentally friendly methods with no residue as the use of non-pathogenic bacteria and plant extracts as biological control agents (Arafat *et al*., 2015; Anuj *et al*., 2019).

It was recently discovered that the electrical and magnetic fields of special frequencies could have a potential impact on the development of bacteria as optional techniques for treating bacterial infections (Liang *et al*., 2006; Ayse *et al*., 2011; Yadollahpour et al. 2014). Macromolecules in the cell wall of bacteria have magnetic activity and using an electromagnetic field affects their pathogenic and growth activity (Segatore *et al*., 2012; Tessaro *et al*., 2015). The activity across the cell membrane of different cells is controlled

using the non-thermal effects of low field intensity ELF-EP. A variety of effects have been observed, including controlling Ehrlich tumors growth in mice (Novikov *et al*., 2009; Fadel *et al*., 2010, 2013, 2017], fungi (Fadel *et al*., 2009), and bacteria (Ayse *et al*. 2011; Fadel *et al*., 2013, 2014; Liu *et al*., 2017; Chen *et al*., 2019). Also, different functional responses, through gene expression (Ibrahim *et al*., 1987, Zalata *et al*. 2015), modification of the function of membrane receptor (Bersani *et al*., 1997, free radical generation (Morabito *et al*., 2010; Wolf *et al*., 2005), and ion homeostasis modulation (Grassi *et al*., 2004; Piacentini *et al*., 2008).

It has been shown that the effects of ELF-EP depend on the type of bacteria, the duration of exposure, and the applied frequency (Benson *et al*. 1994; Kohno *et al*. 2000; Strasak *et al*., 2002, 2005; Fojt *et al*., 2004; Justo *et al*., 2006; Williams *et al*. 2006). Moreover, ELF-EP highest effects have been observed to be at the resonance frequencies (Smith *et al*., 1987; Blackman *et al*., 1994; Fitzsimmons *et al*., 1994; Prato *et al*., 1995; Alipov and Belyaev, 1996; Ali *et al*., 2014; Ali *et al*., 2017; Elkaliuoby *et al*., 2018; Balabel *et al*., 2019).

The present work aims to explore the exposure effectiveness of various frequencies of ELF-EP on the growth ability and the changes in the cell membrane activity of *E. amylovora*.

MATERIALS AND METHODS

Erwinia amylovora growing conditions

E. amylovora strain was kindly given by Dr. Naglaa Moussa Balabel, Director of Potato Brown Rot Project (PBRP). The bacterium was plated on the medium of King's B (KB) agar then incubated at 28°C to 60 hrs. For continuing storage, Luria Bertani broth (LB) is ideal for storing bacterium, 20% glycerol was added, then stored at -80°C (Sambrook *et al*., 1989), also, plated on King's B medium (KB) to revive it.

Pathogenicity test

E. amylovora bacterium was tested for pathogenic on green immature pear buds. A sterile needle loaded with an isolated bacterium was used to puncture four immature pears. Two buds were injected with sterile distilled water as a negative control. Inoculated buds were plated and re-isolated (Naglaa *et al*., 2018). The bacterium pathogenic potential was specified based on the extent of the necrotic lesion or the diameter of the grazing area and oozing according to the Westwood scale (Westwood 1978) as in Table (1).

Table 1. The pathogenic potential of the bacterium on inoculated buds based on the extent of the necrotic lesion or the diameter of the grazing area and oozing according to the Westwood scale.

The degree of grazing area and oozing	Pathogenic potential of the bacterium on inoculated buds
0	no necrosis and no oozing
1	slight necrosis and oozing
2	moderate necrosis and oozing
3	big necrosis and oozing
4	extraordinary necrosis and oozing
5	blackening necrosis and oozing

Pathogenic potential = Σ (Class $\times$ No. of fruits in class) $\times 100$/ (Total No. of fruits $\times$ 5).

Basis of Square Positive Electric pulses

The study was achieved using electronic apparatus to generate a 9V-DC power supply. It was utilized to produce a current of various frequencies in square pulsed form. The square pulses at that point are correlated into DC/DC voltage converter which gives voltage field with intensity (400±25) V. It contains copper plates with an area of 15×15cm^2 and is separated by 20cm.The sample suspension was placed between the plates. The system was produced locally in the physics laboratory of German University in Cairo-Egypt.

Determination of the inhibition frequency

E. amylovora fresh suspension was set up by inoculating 3ml sterile king's B broth with 100 µl of bacterial suspension in a glass test tube closed by a rubber stopper. Eleven groups were prepared (*E. amylovora* suspension with15 sample tubes for each group), one group for the control (unexposed), and 10 groups were treated with various frequencies of ELF-EP source for the interval 0.1 Hz within the range of 0.7 to 1.6 Hz for an exposure duration 30 min. for each frequency at 25°C. At the exposure termination, all samples were added to the incubator at 28°C for 60 hrs during which the bacterial suspension was interrupted for Optical Density measurement (OD) every 6-hour interval (using a sterile king's B broth medium as reference) at wavelength 600 nm using a spectrophotometer (Jenway 6300 UV/visible-UK). Three replicated were carried out for each treatment and the average was calculated. Then, the OD for suspension of bacteria was plotted as a function for the time of incubation to get the growth characteristics curves for all the treatments. For each treatment, growth inhibition difference percentage D% was calculated as shown in equation (1) concerning its control, according to Eq-1. At that point growth inhibition difference percentage against frequency within the range 0.7 to 1.6 Hz was plotted.

D%= [(Average OD (control)–Average OD (exposed)) / Average OD (control)] ×100. (Eq. 1)

DNA analyses

DNA analyses were performed using primers ERIC1R-ERIC2, BOXA1R, and Rep-PCR (McManus and Jones, 1995) (**Table 2).** Suspended one colony of the bacterium in 100µL of sterile water and heated for 5min at 100°C to spin down cell debris and put on ice for 5 min. Amplification was executed in a total volume of 25µL containing 12.5µL of the ready master mix, 1µL from each primer, 7.5µL DNA free water, and 3µL of nucleic acid extract. The following cycling program was used in a thermal cycler (Biometra T-Personal, Germany), the primary denaturation was at 95ºC for 7 min., followed by thirty cycles of denaturation at 94ºC for 1min, annealing at 44, 52, or 53ºC for 1min with REP, ERIC, or BOX primers, respectively, and extraction at 65ºC for 8min with a final extension at 65ºC for 15 min and a soak at 4ºC. PCR products (12µL) were separated onto 2% agarose gels, stained with ethidium bromide (0.5% µg/L), and bands were visualized on a UV trans-illuminator.

Table 2. Primer's characteristics used for analyses of *E. amylovora* by conventional PCR.

Primer name	Primer sequence	Length
REP1R-I	5´-IIIICGICGICATCIGGC-3´	18
REP2-I	5´-ICGICTTATCIGGCCTAC-3´	18
ERIC1R	5´-ATGTAAGCTCCTGGGGATTCAC-3´	22
ERIC2	5´-AAGTAAGTGACTGGGGTGAGCG-3´	22
BOXA1R	5´-CTACGGCAAGGCGACGCTGACG-3´	22

Statistical Analysis

ANOVA was applied to define the significance of the difference among the results that represented as mean ± SE. The significant parameters were compared by using the post-hoc Duncan test at the 5% level. Data statistical analysis was done by SPSS Version 26.0.

RESULTS AND DISCUSSION

Pathogenicity Test

E. amylovora bacterium cultivated on king's B medium for three days at 28°C was examined. **Figure (1)** showed the pathogenic *E. amylovora*. It is important to note that as expressed by oozing from the site of inoculation the infection was started with grazing around the fruit puncture. Prolonged incubation of inoculated buds resulted in relatively variable degrees of fruit blackening and shrinkage (**Fig. 1 Group 1**). The immature pear buds inoculated with sterile distilled water showed no changes (**Fig. 1 Group 2**).

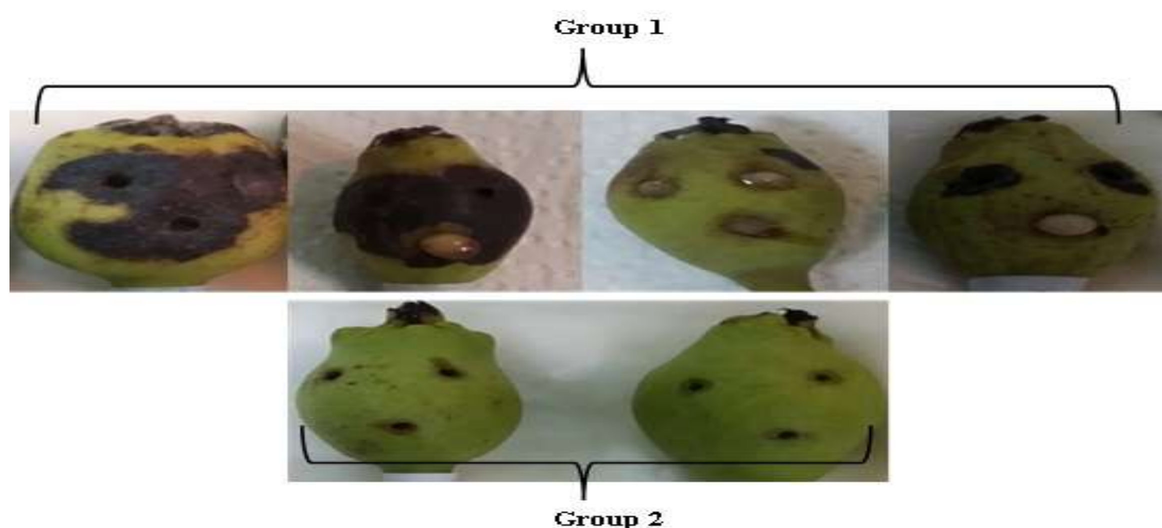

Fig. 1. Immature pear buds produced a blackened area with a droplet of bacterial ooze after inoculation with *E. amylovora* (group (1) and immature pear buds inoculated with sterile distilled water as a negative control (group 2).

Growth Characteristics

Absorbance differences between the treated samples with ELF-EP were in the range of 0.7 Hz to 1.6 Hz for 0.5 hr and the control group is shown in Figure (2). It is evaluated after twelve hrs post-incubation as a reference to its control group. The outcomes demonstrated the resonance peak for cellular growth, decreasing at frequency 1.4Hz. The curves represented in Figure (3) show that the growth difference between exposure at 1.4Hz after 0.5 hr and its control started within the log period at 6th hrs post-incubation and the variance appeared clearly in the exponential growth period after 12^{th}hrs post-incubation and the variance decreased continually until the death growth period at 60th hrs.

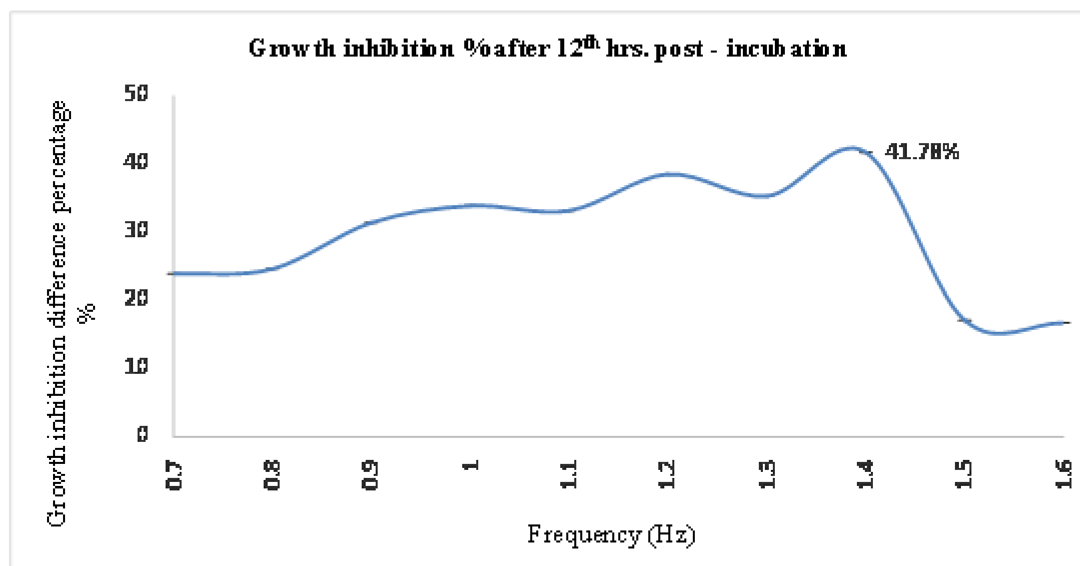

Fig. 2. Differentiation between the inhibition percentages of various frequencies within the range 0.7 to 1.6 Hz on growth of *E. amylovora*.

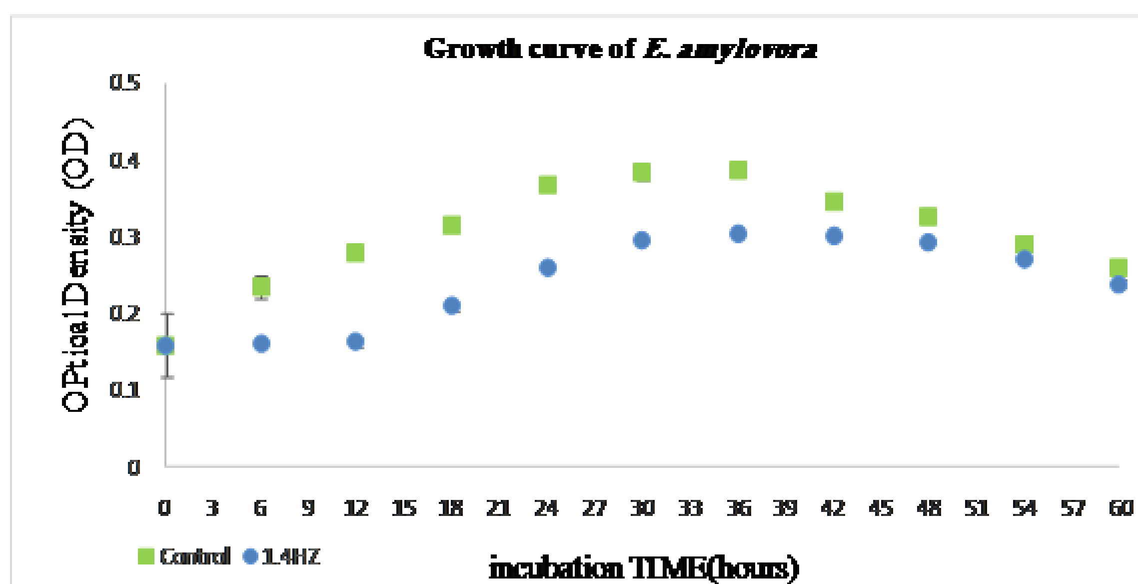

Fig. 3. Growth curves for control (unexposed) and 1.4Hz ELF-EP exposed sample for 0.5hr.

DNA Analysis

Three different molecular markers (Rep PCR, Eric PCR, and Box PCR) were used to differentiate between three different times of exposure to electric pulse (1, 2, 3, 4 hours).

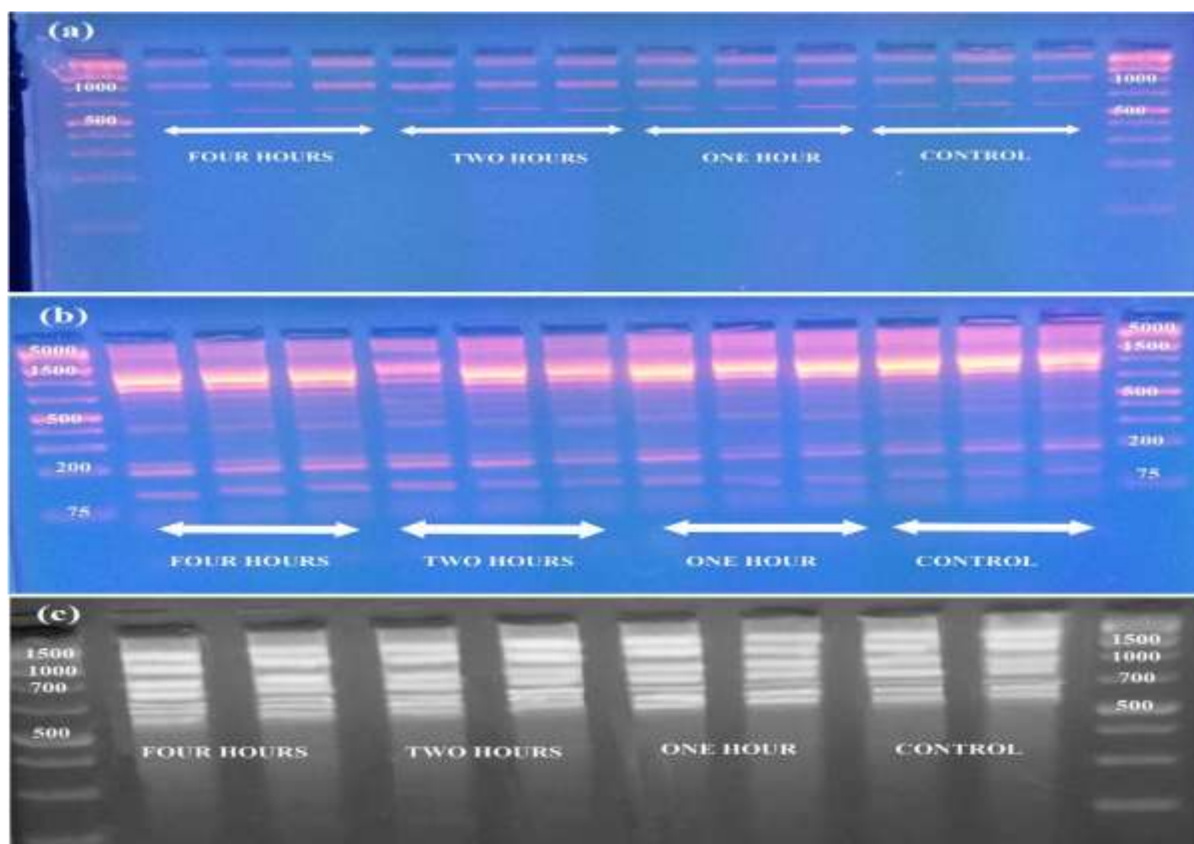

Fig. 4. Rep, Eric, and Box PCR gel electrophoresis of three different times of exposure and control, DNA ladder 100 bp was loaded in the first and end lane.

Electrophoresis pattern, Figure (4 a, b, and c) using Rep, Eric, and Box PCR showed no polymorphism between three different times of exposure to electric pulse compared with control, so the cluster analysis using Dice coefficient divided the treatments with its three times and control into one cluster as in Figure (5). This supports that the treatment by ELF-EP 1.4Hz has no direct effect on the DNA content, but its effect was only on the cell wall activity.

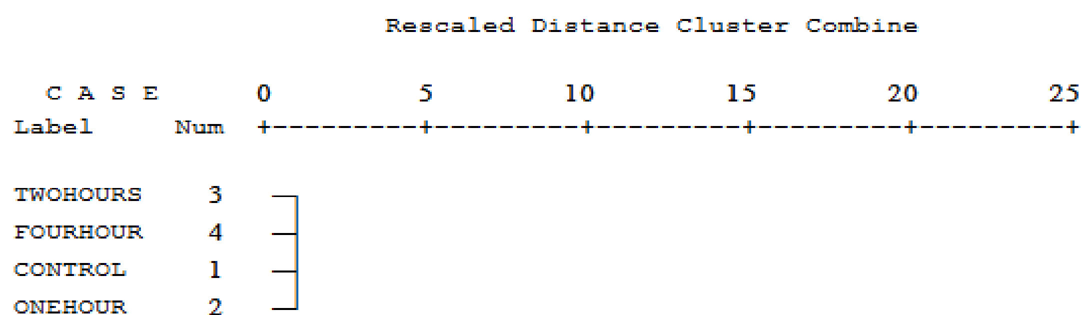

Fig. 5. Dendrogram of cluster analysis based on Rep, Eric, and Box PCR data using Dice coefficient for three different times of exposure and control.

CONCLUSION

It was concluded from the present results that the non thermal ELF-EP technique can successfully inhibit the growth of *E. amylovora* at the resonance frequency. It helps in decreasing the usage of traditional agricultural techniques to fight the fire blight disease. This technique is based on the ability of this non thermal ELF-EP technique to cause alternations in the cell wall structure properties. This is can help in producing healthy crops and save the environment.

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النبضات الكهربائية تقلل نشاط النمو لبكتيريا ايروينياأميلوفيرا

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المستخلص

العمل الحالي يدرس تعرض بكتيريا الايروينيا اميلوفرا لنبضات كهربائية ذات تردد شديد الانخفاض وشدة مجال 400 فولت /متر لترددات مختلفة المدى من 0,7 إلى 1,6 هرتز. والنتائج أظهرت اختلاف بين بكتيريا أيروينيا أميلوفيرا المعرضة لتردد 1.4هرتز والغير معرضة في في منحنى خصائص النمو. والذي أكدته تحاليل الحمض النووي مثبتة أن التغير يحدث في جدار الخلية فقط، حيث أن نتائج تفاعل تسلسل البوليميرز للبريميرز (Rep, Eric, Box) لا تظهر اختلاف بين البكتيريا المعرضة لتردد 1,4 هرتز لأوقات مختلفة والغير معرضة. ومن ثم، خلصت إلى أن معالجة ايروينيا اميلوفيرا بواسطة مجالات النبض الكهربائي للتردد 1,4 هرتز تؤثر على نشاط الخلية والبنية.

الكلمات الدالة: نبضات كهربائية ذات نردد شديد الانخفاض، ايروينيا اميلوفيرا، تفاعل التسلسل البوليمريزي.