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The Effect of Electromagnetic Field on the Growth of *Ralstonia solanacearum* the Causal Agent of Potato Brown Rot Disease

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The effect of the extremely low frequency (ELF) electromagnetic (EM) field on the growth of *Ralstonia solanacearum* was studied where the bacterial suspensions were exposed to different frequencies of square amplitude modulated waves (QAMW) in the range of 0.7-1.2 Hz in steps of 0.1Hz to determine the resonance frequency of growth inhibition. The exposure time varied in a range of 15min-2h. The results indicated that under the best conditions with the electromagnetic field treatment (exposure to1 Hz QAMW for 1 h) the growth of the exposed bacteria was lower than the control one by 66.43%, this difference was highly significant. In addition, the antibiotics susceptibility test using Sulfamethoxazole and Trimethoprim (SXT) and Tetracycline (TE) which act as inhibitors for DNA and protein synthesis was done for both control and exposed bacteria indicated highly significant differences in the susceptibility between control and group exposed to 1.0 Hz QAMW. It could be concluded that the application of ELF EM field could be used to control the disease and in disinfecting potato tubers in storage and before planting.

Keywords: Antibiotic susceptibility, electromagnetic field, growth inhibition, potato, *Ralstonia solanacearum* and resonance frequency.

Potatoes are Egypt's largest horticultural export crop yet, the total value of Egyptian potato exports fell from a peak value of $102.12 million in 1995 to $7.7 million in 2000 mainly due to quarantine restrictions on the potato brown rot imposed by the European Union (EU) which used to account for about 70-90% of Egyptian potato exports (Kabeil *et al.*, 2008).

Potato brown rot, caused by *Ralstonia solanacearum* race 3 biovar 2, (which is classified as one of the world's most important phytopathogenic bacteria due to its lethality, persistence, wide host range and broad geographic distribution), is a serious endemic disease in the Nile Delta of Egypt. It is a quarantine disease in the EU, and export of potatoes from Egypt is restricted to pest-free areas in the desert. Race 3 biovar 2 is the dominant race in mountainous regions in South America (Andes), where the potato and possibly this race of *R. solanacearum* originate, at higher altitudes in other tropical areas and also in the Mediterranean

basin, including Egypt and more recently even in NW Europe. Potato losses up to 75% due to the bacterial wilt have been recorded in many countries (Cook and Sequeira, 1994). Because of the frequent occurrence of brown rot in Egyptian potatoes exported to European markets, its endemic presence in the Nile Delta area and a possible threat via contamination of irrigation water that became contaminated by waste of industries processing Egyptian potatoes, the European Commission (EC) took restrictive measures. In its decision, Anonymous (1998) demanded that potatoes should be produced in so-called pest free areas (PFA's) according to FAO standards, where the disease should be known, not to occur via testing (Anonymous, 2005; Elphinstone *et al.*, 1998 and Janse, 1996).

Many trials have been carried out all over the world to control the disease without much success. No promising control of brown rot was achieved using antibiotics (Habashy *et al.*, 1993), soil fumigants (Weingartner and Shumaker, 1988), chemical control (Murakoshi and Takahashi, 1984) or breeding of resistant varieties (Hartman and Elphinstone, 1994; Mendoza, 1994; Fock *et al.*, 2001 and Lopez and Biosca, 2004). Moreover commercial chemicals have generally proven to be ineffective in controlling the pathogen and are not recommended as a means of control (Denny, 2006) due to the increasing demand for low-input and organically produced products since the last ten years because of fear of the hazardous effects of pesticides and chemical residues both in Europe and Egypt (Sylvander and Le Floc'h-Wadel, 2000; Parrott and Kalibwani, 2004). Also biocontrol agents such as *Pseudomonas fluorescens*, *P. glumae*, *Burkholderia cepacia*, *Bacillus* sp., *Erwinia* sp. and a Hrp-mutant of *R. solanacearum* were found to be relatively ineffective in the control of *R. solanacearum* populations under natural conditions (Lopez and Biosca, 2004 and Ran *et al.*, 2005). The failure probably results from the inability of the introduced biocontrol agent to establish itself and to produce the desired compound under the stress of competition by the native microbial community.

Recently, the efforts were devoted to control cellular activities by using electromagnetic waves of very low field intensity and frequencies which resonates with bioelectric signals generated during a particular metabolic activity. These trials succeeded to control the growth of Erlich tumours metastasis in mice (Fadel *et al.*, 2010), bacterial cells and fungi (Fadel *et al.*, 2009).In the present work, a trial was made to find out the resonance frequency of the electromagnetic waves that can inhibit the activity of *R. solanacearum* and its ability to make division, as well as to investigate the changes that may occur at the molecular level as a result of exposure to ELF-EMFs.

Materials and Methods

1- Culture conditions of the R. solanacearum test strains:

R. solanacearum isolate was obtained from the Plant Pathol. Dept., Fac. of Agric., Cairo Univ., Egypt. Physiological and biochemical tests were carried out according to the methods described by Hayward (1964) and bacteriological characteristics have been done according to Bergey's manual of systematic bacteriology (Palleroni, 1984). *R. solanacearum* isolate were grown on standard

laboratory nutrient broth media containing: (1.5g beef extract, 5g glucose, 5g peptone, and 5g NaCl) per 500ml distal water. The bacterial culture was produced by suspending one colony for 48h at 28°C. For agar medium, agar was added, then the mixture was heated to dissolve agar and sterilized by autoclaving from 121-124°C for 15 minutes. For maintaining the bacterial strain, the bacterial culture was plated on a fresh nutrient agar plate and placed in an incubator at 28°C, this procedure was repeated every other day.

2- Measurements of bacterial growth:

Bacterial growth was determined by measuring the optical density at 600 nm (OD_{600}) of *R. solanacearum* suspensions. The bacterial suspension was prepared by adding 250µl of bacterial culture to 10ml of sterilized nutrient broth. The suspension then incubated at 28°C and every 1hr the incubation was interrupted for absorbance measurements via JENWAY 6405 UV/Visible (U.K.) spectrophotometer (using nutrient broth as a blank). The absorbance of bacterial suspension then plotted as a function of incubation time. To correlate OD_{600} values with colony forming units (CFU) a calibration curve was established by using plate counting technique (Atlas, 1995).

Since that, the relationship between bacterial concentration and absorbance begins to deviate from linearity when the bacterial culture becomes so turbid due to secondary scattering as the concentration of bacteria increases; a correction of the deviation from the ideal Beer-Lambert relationship was calculated.

3- ELF EM field application:

Samples of *R. solanacearum* suspension were exposed to different frequencies QAMW. The modulating waveform was square generated by synthesized arbitrary generator type TTi TGA 1230. the carrier frequency was 10MHz sine wave generated by a wave generator model AFG 310 manufactured by Sony Tectonics, Japan, The amplitude of the wave carrier was $10V_{pp}$ and the modulating depth was ±2V (Fig. 1). Different groups of *R. solanacearum* were exposed to different frequencies of the QAMW through two parallel cupper electrodes of separation distance 1cm.

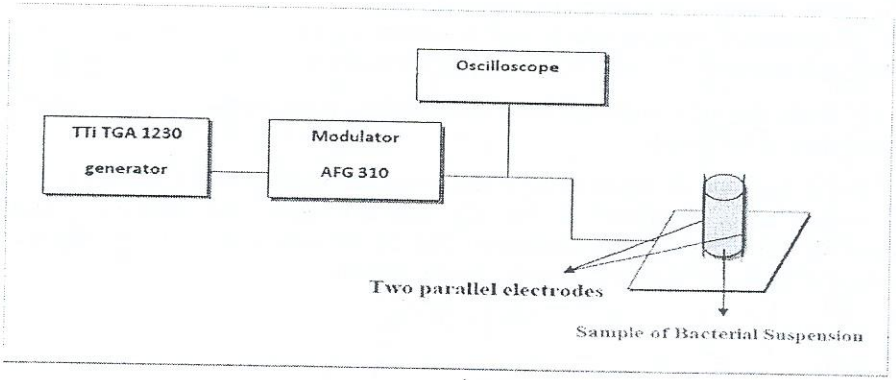


Fig. 1. Circuit block diagram for the exposure facility of the bacterial culture.

A fresh bacterial suspension of *R. solanacearum* has concentration of approximately 10^{12} CFU/ml was divided into seven groups; one group kept as control and the other groups exposed to different frequency QAMW in the range of 0.7 to 1.2 Hz in steps of 0.1 Hz for 1hr in order to determine the resonance frequency of growth inhibition. Field exposure took place in the sample incubator at 28°C and every 1hr the incubation was interrupted for absorbance measurements. Also for determining the optimum exposure time, seven groups of *R. solanacearum* suspension was prepared; one group kept as control and the other groups exposed to QAMW at resonance frequency of growth inhibition for periods of 15, 30, 45, 60, 90 and 120 min. then the growth measurements was done every 1hr.

4- Antibiotic susceptibility test:

Antimicrobial susceptibility testing is a standardized testing method used to measure the effectiveness of antibiotic agents on pathogenic microorganism. The antibiotics used in the study were Amikacin (AK) and Tetracycline (TE) which act as inhibitor for protein synthesis, Ciprofloxacin (CIP), Sulfamethoxazole and Trimethoprim (SXT) acting as inhibitor for DNA synthesis and Ampicillin (AM) that causes deterioration of cell membrane. Discs as well as zone reading chart were supplied by BBL™ (Zimbro *et al.*, 2003). Antimicrobial susceptibility test was carried out and performed by the procedure outlined by Anonymous (1985).

Two groups of *R. solanacearum* suspension were used; one group exposed to QAMW at inhibiting resonance frequency for a period of 1hr and the other kept as control. At the end of exposure period, samples of exposed and control groups were used to inoculate nutrient agar plates, then the antibiotic discs were placed on the agar surface. The inoculated plates then incubated at 28°C for 48hr. then the diameter of each inhibition zone was measured with a ruler and compared with the diameter supplied by BBL™.

5- Calculations and statistical methods:

Data from bacterial growth studies and antibiotic susceptibility test were compared for statistical significance using a two tailed Student t-test with $P<0.05$ considered significant.

Results

The growth dynamic for control and exposed groups:

The correction formula which used to correct for linearity at high absorbance readings is given by: $A_c = 4.92\text{-}6.513\ (0.570\text{-}0.234\ A_u)½$

Whereas: A_c is the corrected absorbance and A_u is the uncorrected (measured) absorbance of the sample.

The growth curve characteristics and count-absorbance calibration curve of *R. solanacearum* illustrated in Fig. (2a and b). Figure (2b) shows a linear dependence of the corrected absorbance (A_c) on the number of CFU/ml (N), the linear plot in the figure was represented by the formula:

$$N = 6 \times 10^{13}\ A_c$$

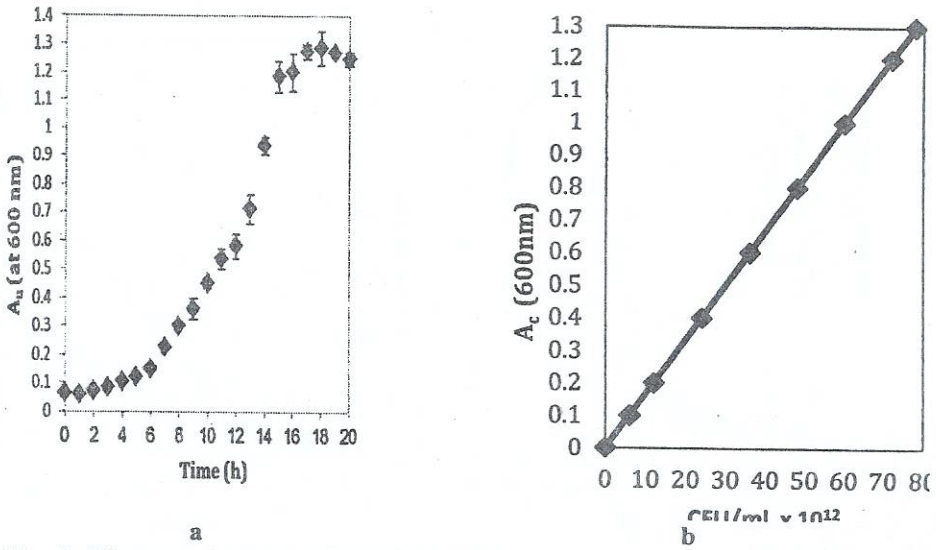


Fig. 2. The growth curve characteristics (a) and count-absorbance calibration curve (b) of *R. solanacearum*.

An application of the seven different frequencies of QAMW (0.7-1.2 Hz) on *R. solanacearum* suspension showed a significant effect regarding the stimulation or inhibition of bacterial growth (Fig. 3a, b, c and d). Figure (3a, b, and c) illustrates the growth curves of *R. solanacearum* cells after being exposed to QAMW for 1 h at frequencies 0.7, 1.0 and 1.1 Hz, respectively, as compared with control group. It is clear from the figure that an enhancement of the bacterial growth occurred for samples exposed to a frequency of 0.7 Hz QAMW. In contrast, an inhibition of bacterial growth occurred after exposure to 1.0 Hz QAMW, while the exposure to 1.1 Hz QAMW almost has no effect on bacterial growth.

Figure (3d) illustrates the differences in absorbance between groups exposed to QAMW in the range 0.8 to 1.2 Hz for 1 h and control group at 16^{th} and 18^{th} hours of incubation. The data in the figure indicate a highly significant growth inhibition occurred after exposure to 1.0 Hz QAMW for 1 h ($P < 0.00001$), which reflects that the 1.0 Hz QAMW is the resonance frequency of growth inhibition for *R. solanacearum*.

The effect of different exposure times to 1.0 Hz QAMW on the growth characteristics of *R. solanacearum* is illustrated in Fig. (4), which shows a histogram relating the percentage of growth inhibition after different exposure periods at 15^{th} h of incubation. It is clear from the figure that the exposure of *R. solanacearum* suspension to 1.0 Hz QAMW for 1 h causes maximum growth inhibition by 66.43% with respect to control while for the 0.25, 0.5, 0.75, 1.5 and 2 h exposure periods, the percentage of growth inhibition was 9.87, 26.6, 46.49, 31.46 and 31.55%, respectively.

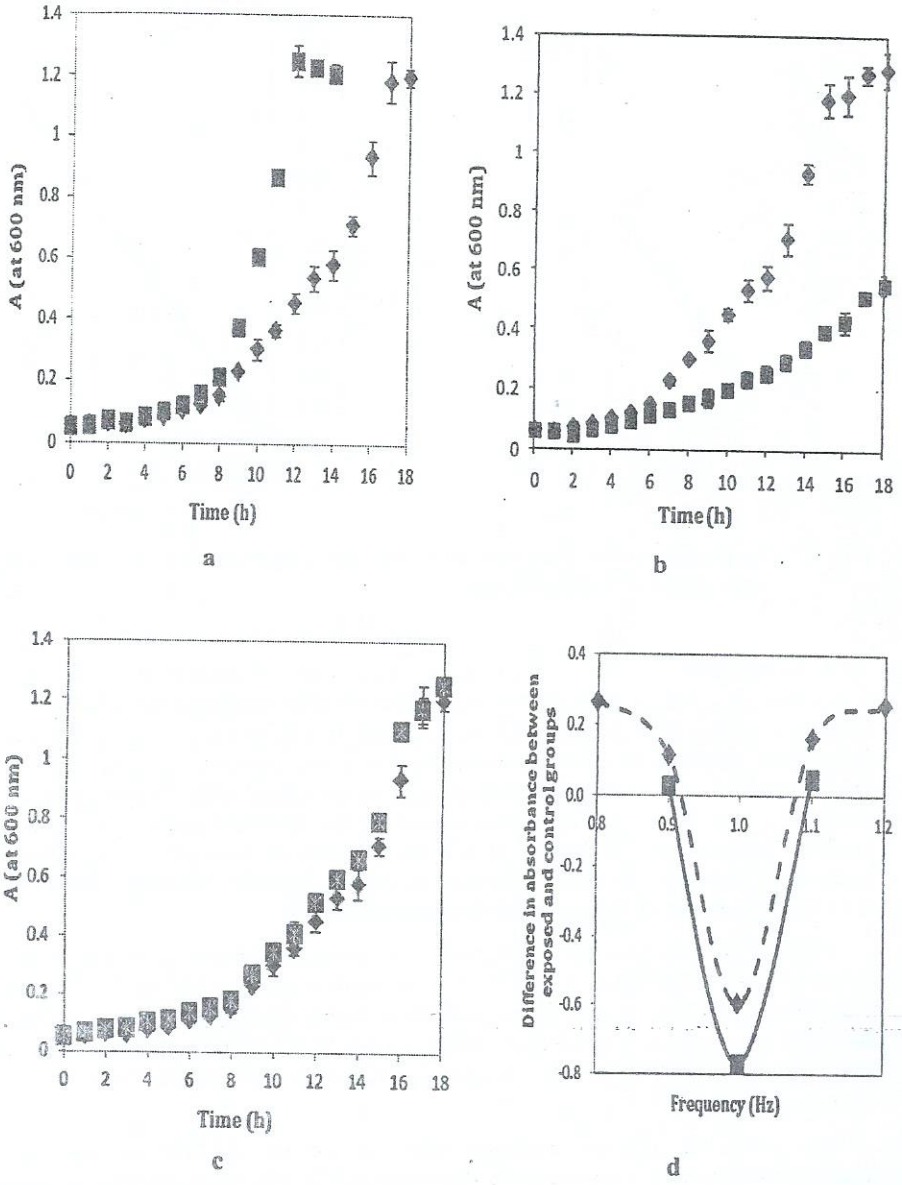


Fig. 3. The growth curves of *R. solanacearum* groups exposed for 1 h to QAMW (■) at frequencies 0.7 Hz (a), 1.0 Hz (b) and 1.1 Hz (c) compared with control group (♦).The difference in absorbance between groups exposed to QAMW in the frequency range from 0.8 to 1.2 Hz for 1 h and control group as a function of frequency at 16th (dash line) and 18th (solid line) hours of incubation (d).

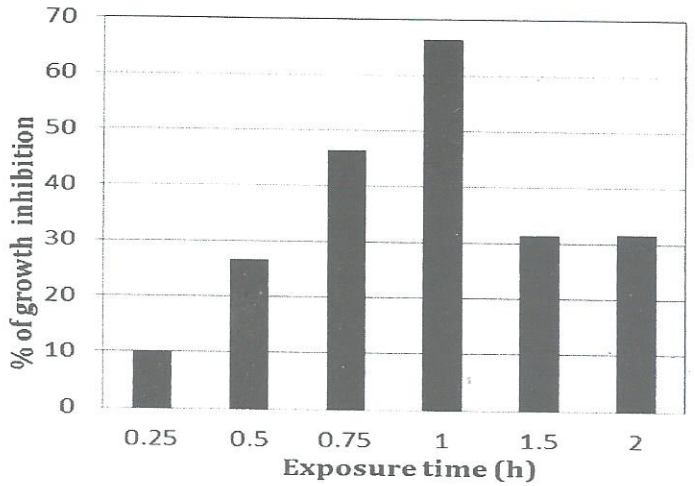


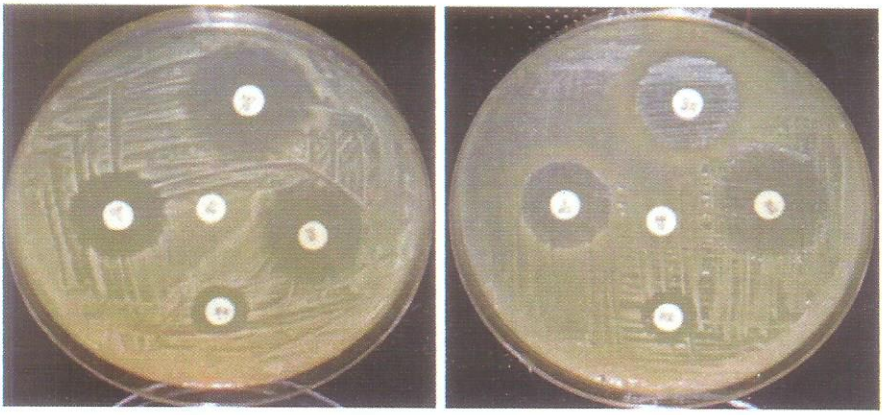
Fig. 4. The percentage of growth inhibition after different exposure periods to 1.0 Hz QAMW at 15th h of incubation.

Effects of antimicrobial agents:

The results of antibiotic susceptibility test of *R. solanacearum* for control group and group exposed to 1.0 Hz QAMW for 1 h in Table (1) showing that both groups resisted the effect of AK, but the group exposed to 1.0 Hz QAMW was more resist to AK effect than the control. On contrast the two groups were highly susceptible to the effect of TE but the group exposed to 1.0 Hz QAMW had more susceptibility to TE than control, $P<0.0005$ which is highly significant. Regarding to the antimicrobial agents CIP and SXT which act as inhibitors for DNA synthesis, the control group showed susceptibility to the effect of CIP while the group exposed to 1.0 Hz QAMW shows intermediate response. For SXT, both groups showed high susceptibility to its effect but the control group was more susceptible than exposed group, the susceptibility difference between them was highly significant ($P < 0.001$). The antibacterial agent AM did not have any effect on both groups. The inhibition zones of the different antimicrobial agents for control group, group exposed were shown in Fig. (5a and b).

Table 1. The mean inhibition zone diameter of different antimicrobial agents effect on control group and exposed group of *R. solanacearum*

Antibiotic / Sample	Mean inhibition zone diameter (mm)				
	AK	TE	CIP	SXT	AM
Control	14±1	23±1.5	21±1	27±0	0±0
1Hz QAMW	11.5±0.5	30±0.5	19±0.5	21±1.5	0±0



a b

Fig. 5. Inhibition zones of the different antimicrobial agents for a) the control and b) group exposed to 1Hz QAMW.

Discussion

Several studies focus on the effect of electromagnetic fields on the growth of bacteria. Stepanian *et al.* (2000) demonstrated that *Escherichia coli* cells lost capacity for division and unable to form the colonies on the solid media after exposure to the static magnetic field and extremely low frequency electromagnetic field. Fojt *et al.* (2004) observed a decrease in the number of viable bacteria following exposure to low-frequency electromagnetic fields at 50 Hz. Lipiec *et al.* (2004) reported that the application of oscillating magnetic field pulses might be used in disinfecting agricultural products and food. Strašák *et al.* (2005) observed a decrease of optical densities for different bacterial strains exposed to a 50 Hz electromagnetic field at room temperature. David *et al.* (2006) reported that application of the ELF magnetic field allowed the deviating of the metabolic pathway of *Lactococcus lactis* subsp. *lactis* in order to intensify or to inhibit the nisin production. Cellini *et al.* (2008) demonstrated that the exposure of *E. coli* to a 50 Hz electromagnetic field acts as a stressing factor leading to phenotypical and transcriptional changes.

In previous studies only one type of field was investigated regardless the effect of frequency and resonance phenomenon. However, in this study we compared the effect of different ELF electromagnetic field on growth/growth inhibition of *R. solanacearum* in order to determine the resonance frequency of growth inhibition. It was found that the exposure of *R. solanacearum* suspension to 1.0 Hz QAMW for 1 h causes growth inhibition by 66.43% while a significant increasing in bacterial growth occurred after exposure to 0.7 Hz QAMW for 1 h which means that the effect of ELF electromagnetic field on bacteria neither bactericidal (killing the bacteria) nor bacteriostatic (blocking their growth during the exposure). The underlying biochemical effects of electromagnetic fields have not been clarified until now (Cellini *et al.*, 2008). It may be presumed from the present results that the

effects of ELF-EMF on bacterial growth (*R. solanacearum*) are due to the interference of these fields (according to the frequency) with the bioelectric signals generated from physiological functions of bacterial cell. The results of these interference reactions depend on the mode of interference which may lead to inhibition (destructive mode) or enhancement (constructive mode) to the running physiological process. All these may lead to changes in DNA and proteins structures.

This assumption could be confirmed by the results of antibiotic susceptibility test, where the group exposed to 1.0 Hz QAMW had more susceptibility to TE than control which means increasing in membrane permeability to TE (Tritton, 1977), under the effect of ELF electromagnetic field. This was in agreement with Costa *et al.* (2002) where he reported that high and low frequency electromagnetic radiations were able to change the kinetics of the potassium and sodium channels in a squid giant axon model.

In contrast it was observed that the control group showed more susceptibility to SXT than the group exposed to 1.0 Hz QAMW, this means that the enzymatic activity of dihydropteroate synthetase and dihydrofolat reductase; the binding sites of SXT (Kalkut, 1998); reduced under the effect of ELF electromagnetic field leading to lower susceptibility of exposed group to SXT. this assumption is also supported by decreasing in growth after the exposure to 1.0 Hz QAMW where the decreasing of dihydropteroate synthetase and dihydrofolat reductase enzymatic activity affect the formation of tetrahydrofolic acid, thymidine synthesis and subsequently DNA and proteins synthesis (Pattishall *et al.*, 1977). Ravera *et al.* (2011) also found that the exposure of bovine lung membrane carbonic anhydrase (CA) to ELF electromagnetic field caused a decrease in the enzymatic activity of CA by 17%.

These changes between control and exposed proved that the DNA sequences have been changed under the effect of ELF electromagnetic field in a way that a new binding sequences are appeared. Distinct changes in gene expression have been detected also in cells after exposure to either radio-frequency electromagnetic, ELF and static fields by Maercker (2005) and Lupke *et al.* (2006).

These DNA mutation and genetic alteration caused by the bacterial exposure to 1.0 Hz QAMW results in changes in the translated proteins and enzymes that the mutated gene codes for and this subsequently may be effecting bacterial growth (Van der Wolf *et al.*, 2004; Salanoubat *et al.*, 2001 and Lee *et al.*, 2001).

ELF electromagnetic field can affect the bacterial growth in vitro in both ways stimulation and inhibition depending on the frequency in addition that it causes a genetic alteration in the DNA, so it could be used in controlling the growth of pathogenic *R. solanacearum*.

Our future work will involve an in vivo study on the effect of 1.0 Hz QAMW on *R. solanacearum* growth in potato in addition to determining if 1.0 Hz QAMW effecting the cell membrane electrical properties or morphology. Also, comparing between the effect of 1.0 Hz QAMW and 1.0 Hz ELF magnetic field on the growth inhibition of *R. solanacearum*.

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تأثير المجال الكهرومغناطيسي علي نمو بكتريا رالستونيا سولاناسيرام المسببة لمرض العفن البني في البطاطس

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تمت دراسة تأثير الترددات المنخفضة جداً من المجال المغناطيسي علي نمو بكتريا رالستونيا سولاناسيرام حيث تم تعريض معلق البكتيريا الي موجات كهرومغناطيسية معدلة السعة مربعة في حيز تردد من ٠,٧ الي ١,٢ هرتز لتحديد التردد الرنيني لإيقاف نمو البكتيريا. بعد ذلك تم تعريض الخلايا البكتيرية للموجات الكهرومغناطيسية عند التردد الرنيني لفترات مختلفة من ١٥ الي ١٢٠ دقيقة. وقد اظهرت النتائج ان هناك نقص كبير في نمو بكتيريا رالستونيا سولاناسيرام نسبته ٦٦,٤٣% عند تعرضها لتردد ١هرتز من الموجات الكهرومغناطيسية معدلة السعة المربعه لمدة ٦٠ دقيقه و كان هذا الإختلاف كبير المعنوية.

علاوة علي ذلك، لوحظ حدوث تغييرات في حساسية رالستونيا سولاناسيرام تجاه المضادات الحيوية بعد التعرض لتردد ١ هرتز من الموجات الكهرومغناطيسية معدلة السعة المربعه لمدة ٦٠ دقيقة. حيث اصبحت البكتريا المعرضه أكثر حساسية لتأثير التتراسيكلين في حين انها اصبحت اقل حساسية لتأثير سلفاميثوكسازول- تريميثوبريم مما يدل على حدوث تغييرات في البروتينات الموجودة داخل الخلايا البكتيريه والتي تعمل عليها هذه المضادات الحيوية وذلك نتيجه التعرض للمجالات الكهرومغناطيسية.

وبناء على ماسبق يتضح لنا أنه يمكن استخدام المجالات الكهرومغناطيسية ذات الترددات شديدة الانخفاض عند التردد الرنيني وذلك للسيطرة على مرض العفن البنى للبطاطس وايضاً امكانية تعقيم المخازن وقبل الزراعة.