

## Control of *Sclerotium Cepivorum* (Allium White Rot) Activities by Electromagnetic Waves at Resonance Frequency

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**Abstract:** *Sclerotium cepivorum* fungi were isolated from infected onion roots, purified then cultured on PDA medium in Petri dishes. The samples in the dishes were exposed to square amplitude modulated waves (SAMW) from generators with constant carrier frequency of 10 MHz and amplitude 20Vpp and modulating depth  $\pm 2V$  through the use of two parallel copper plates fixed on the two opposite sides of the Petri dishes. Each sample was to one specific modulating frequency of the SAMW and the mycelium growth rate and the number of sclerotia produced in each dish were followed. The results indicated that no sclerotium was produced in samples exposed to 20 Hz SAMW for two hours. Therefore, the changes in the DNA molecular structure of these samples as a result of exposure to 20 Hz were studied and compared with control (unexposed) through the use of PCR and primers. The results indicated the appearance of a new band in the DNA at 190 pb by the primer OPAO3 which can be considered as markers for genetic alterations in the DNA which control sclerotia production.

**Key words:**

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### INTRODUCTION

Fungal infection of crops have both economical and health impacts. Therefore, it is considered as one of the greatest problems facing agricultural health studies. Many fungi may completely destroy or at least devalue the crops (Christensen and kaufman, 1969). In addition some of these fungi may produce serious carcinogenic mycotoxins (Bhatnagar *et al.*, 2002 & Smith and Ross, 1991). Therefore, many fungicides were developed to overcome this problem. Further studies showed that synthetic fungicides themselves may be considered as a great problem due to its possible health effects which may be direct through long-term dietary administration of crops contaminated with such fungicides (Svechnikov *et al.*, 2005), or indirect through the induction of secondary metabolites with adverse health effects (Havlova *et al.*, 2006)

In the recent years, public pressure has increased to reduce the use of synthetic fungicides in agriculture. Concerns have been raised about both the environmental impact and the potential health risk related to the use of these compounds. Therefore, considerable efforts have been made towards the development of alternative crop protectants (Santino *et al.*, 2005). Ionizing radiation is one of the alternatives being used to reduce fungal and microbial contamination of crops (Smolianina *et al.*, 2006 & Mintier and Foley, 2006 & Dhokane *et al.*, 2006 & Shahhoseini, 1998). But still ionizing radiation can be used only for limited quantity of the crop and can not be applied in the land of field. Some studies showed that Electromagnetic fields may affect microbial growth (Aronsson *et al.*, 2005 & Elez-martinez *et al.*, 2004) which may be used to control the growth of fungi.

One of the most serious soil-borne fungus is *Sclerotium cepivorum* Berk, which is the causal agent of *allium* white rot disease (Entwistle, 1990a), one of the most serious diseases of onions (*allium cepa* L.) (Metcalf and Wilson 1999). This disease can reduce yields to uneconomic levels in four years of successive onion crops (Coley-smith, 1987). The fungus penetrates the root epidermis then invades the cortical parenchyma (Abd El-Razik *et al.*, 1973) Infected plants suffer from water stress and often die prior to harvest or rot in storage (Entwistle, 1990b). The pathogen persists in the soil in the absence of host plants as sclerotia and can survive in this form for more than 20 years. (Coley-Smith 1990a). Following their production, Sclerotia are constitutively dormant for 1-3 months and will then only germinate in the presence of host plants (Coley-Smith *et al.*, 1987). The stimulus for germination is the exudation of alk(en)yl cysteine sulphoxides are metabolized by the soil microflora to produce volatile thiols and sulphides which trigger the dormant sclerotia to germinate (Coley- Smith and Parfitt, 1986). The

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efforts of our research group in the last few years were devoted to control cellular activities by using electromagnetic waves of frequencies which resonates with a bioelectric signals generated during a particular metabolic activity. These trials succeeded to control the growth of Erlich tumors in mice (Fadel *et al.*, 2005 and 2009) and bacterial cells (El Hag, 2005).

In the present work, a trial was made to find out the resonance frequency of the electromagnetic waves that can inhibit the activity of *Sclerotium cepivorum* and its ability to produce sclerotia, and to investigate the genetic alterations that may occur as a result of exposure.

## MATERIALS AND METHODS

### ***Isolation and Identification of Pathogens:***

Infected roots with *Sclerotium cepivorum* were sectioned and surface sterilized in 10% commercial bleach (0.525% sodium hypochlorite) for 30 seconds. The samples were washed thoroughly in sterilized water, dried between sterilized filter papers, then transferred onto PDA (Potato dextrose Agar) medium in petri dishes which were incubated at 28 - 30°C for five to seven days. The fungi were isolated purified by hyphal tip technique (Brown, 1924) and kept on slant of PDA in test tubes at 5°C.

### ***Culture Characteristics:***

Agar disks (7mm in diameter) cut from the margin of actively growing colonies on PDA medium were transferred to 9 cm petri dishes containing 20 ml of PDA. Five dishes of each isolate were incubated for one week at 25°C in the dark. Colony diameter of radial growth was recorded every day according to Lipps and Herr (1982). Presence or absence of sclerotia and radial growth rate were determined for all isolates.

### ***Exposure Facility:***

Samples of Scl. Cep. inoculated on PDA were exposed to different modulating frequencies of amplitude modulated waves (AMW). The modulating waveform was square and the carrier frequency was 10 MHz sine wave. The wave carrier was generated by a wave generator model AFG 310 manufactured by Sony Tektronics, Japan, and the modulating wave was generated by synthesized arbitrary generator type TTi TGA 1230. The amplitude of the wave carrier was 20 V<sub>pp</sub> and the modulating depth was ±2V.

The samples in Petri dishes were exposed to the AMW through two parallel copper disk electrodes, each of diameter 8 cm.

During our trials to find out the resonance frequency of the modulating waves, samples were exposed for 2hr (single dose) to each frequency. After finding the resonance frequency a trial was made to evaluate the most effective exposure time and protocol effects of. In this part of the study samples were exposed for different exposure times (2 hr and 4 hr) with different exposure protocols (single dose and repeated dose for 3 successive days). Triplets of samples were used in each experiment.

After exposure the mycelium growth rate and the number of sclerotia produced in each dish were followed.

### ***Molecular Analysis***

#### ***Polymerase Chain Reaction (PCR):***

##### ***Mycelium Preparation:***

Isolates were grown in 100 ml of PD liquid medium (extract of 200 gm of potatoes + 20 gm dextrose per liter) for 7 days at 25°C. Mycelia were harvested by filtration, air dried and ground to a fine powder in liquid nitrogen using a mortar and pestle.

##### ***DNA Isolation:***

Total DNA was isolated from the mycelial powder using the procedures of Lee and Tylor (1990).

##### ***Primers:***

Four of ten-oligonucleotide primers were successfully used to amplify DNA segments {OPA03 (AGTCAGCCAC), OPD05 (AGGGGTCTTG), ITS2(GCTGCGTTCTTCATCGATGC) and TS3(GCATCGATGAAGAACGCAGC)}. Ten-base oligonucleotide primers (random primers) i.e., OPA03 and OPD05 were purchased from Operon Technologie Inc Alameda, Ca; kits A, O and P were used. White *et al.* (1990) designed a series of PCR primers based on highly conserved regions of rDNA (i.e., ITS2, ITS3, ITS4 and ITS5). These primers are applicable to a variety of organisms and can be used as "universal" primers to amplify

the DNA regions encoding the nuclear small subunit rRNA (SrDNA) and the transcribed spacer regions between the small and the large subunit rDNAs. These two regions of rDNA evolve at different rates (White *et al.*, 1990).

#### ***Amplification Conditions:***

Aqueous amplification reactions were made in a total volume of 25  $\mu$ l, containing 10 mM Tris-HCL (pH 8.3), 50mM KCL, and dTTP, 0.4  $\mu$ M of primer, 50 ng of genomic DNA, and 0.5 unit of Taq polymerase under 60  $\mu$ l of mineral oil. DNA was amplified in a DNA thermal cycler programmed as follows: one cycle for 2 min at 94 C°; followed by 40 cycles of 1 min at 93 C°, 1 min at 36 C° and 1 min at 71 C° and one cycle for 5 min at 71 C°. Fifteen  $\mu$ l of the amplification products were electrophoresed on 1.4% agarose gels stained with ethidium bromide (1% aqueous solution) and photographed under UV radiation.

The RAPD technique described by Williams *et al.* (1990) was used as a starting point for setting up a standard protocol suitable for *scl. cepivorum* isolates and for detecting the genetic variations or changes in the DNA among the control and exposed isolates.

#### ***Statistical Analysis:***

The statistical methods and analysis for evaluation of the results were done by calculating arithmetic means and standard deviations for growth diameter and the number of Sclerotia produced after 10 days of fungal culture. All these measurements have been done for all isolates of all groups. The average readings of three plates were used to calculate the means and the standard deviations for each group. The data presented here shows that there is a high significant difference (P-value <0.001) between the isolates exposed to 20 Hz and that of the control group as well as the isolates exposed to other frequencies.

## **RESULTS AND DISCUSSIONS**

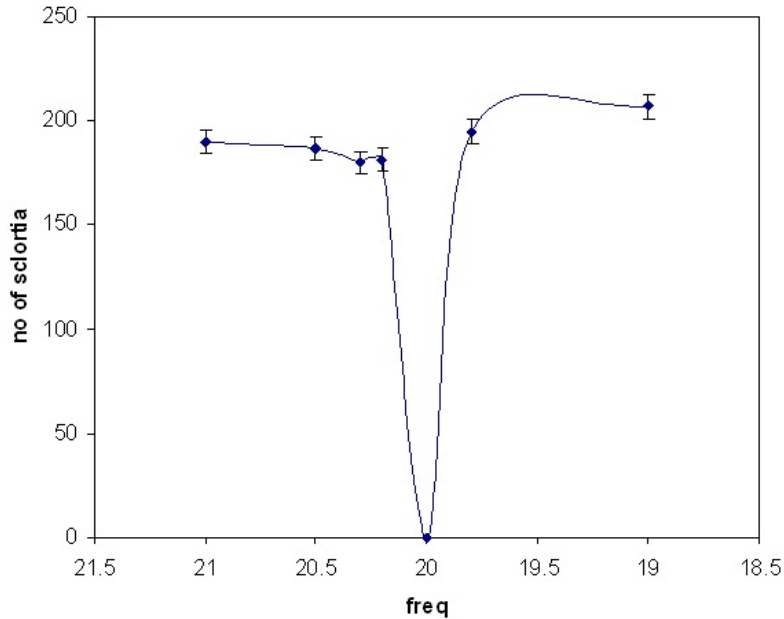
#### ***Results:***

Table (1) shows the the variation of the growth diameter of mycelium and sclerotia production of *scl. cepivorum* isolates as a function of the frequency of the square amplitude modulated waves (SAMW) applied. It is clear from the table that there is no dependence of the mycelium growth diameter on the modulating frequency of the maximum effective waves, However the data indicates that there is no Sclerotia production when the samples were irradiated at a frequency of 20 Hz for a single exposure of two hours. For more microscan of the irradiation frequency the experiment was repeated for different samples after being irradiated for a period of two hours and the scan step was done at intervals of 0.1 Hz (Table 2). Fig. (1) illustrates the variation of the sclerotia production as a function of the applied field frequency. The results indicate that there is a resonance frequency of 20 Hz at which no sclerotia were produced. Fig. (2) shows a photograph for the sclerotia produced on PDA medium 10 days post inoculation for control and irradiated samples with square amplitude modulated waves of frequency 20 Hz for 2 hr. The photograph shows no sclerotia could be noticed for the 20 Hz exposed samples. Statistical evaluations for the data of the different groups compared to that exposed to 20 Hz show that there are very high significant difference (P-value <0.0001) between the compared groups.

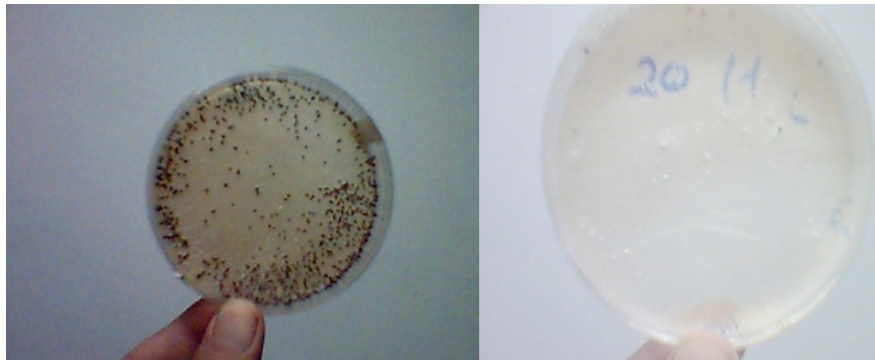
In a trial to investigate the effect of exposure time on the studied parameter, the samples were exposed to 20 Hz SAMW for a single exposure of 2 hrs or 4 hrs in one day and for three successive days at a rate of 2 hrs / day or 4 hrs / day . The results are given in Table 3. The data in the table indicates that there is no dependence of the measured parameters on the exposure time or the exposure protocol of the samples. Genetic variations or changes in the DNA among the control and exposed isolates were detected by RAPD technique. We noticed that the temperature of the template DNA, Mg cl<sub>2</sub>, Taq polymerase and the thermal cycling profile were varied independently to define reaction conditions which generate reproducible and scorable RAPD profiles.

Fig. (3) shows that, out of 10 primers screened only four provided informative RAPD pattern for all isolates, showing one to ten fragments per isolate (lane) in the size range of 0.25-3.5 Kb. Figs. (3 and 4) show that RAPD profiles exhibiting distinct fragments for all exposed isolates and control. One of the four primers, OPAO3, (AGTCAGCCAC) could distinguish between isolates exposed to 20 Hz and the control one. Only one primer (OPA03) gave a band (190pb) which distinguished isolates exposed to 20 Hz from control isolate (Fig. 3).

This discriminating band was present only in the lane of the isolate exposed to 20 Hz and absent in the other lanes (isolates exposed to 1, 5, 10, 15 and 25 Hz) (fig. 4);



**Fig. 1:** Number of sohlortia produced as a function of frequency of the field applied.



**Fig. 2:** Sclerotia production of Isolates of *Sclerotium cepivorum* are on potato dextrose agar after 10 days of fungal culture.

(A): Isolate of *Sclerotium cepivorum* is without any exposure.

(B): Isolate of *Sclerotium cepivorum* exposed to 20 Hertz (Hz) SAMW for 2 hrs (single dose).

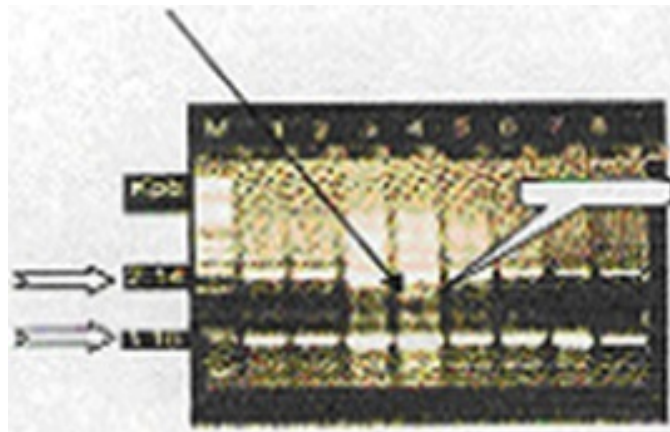
**Table 1:** Growth diameter of mycelium and sclerotia production of *Sclerotium cepivorum* Isolates exposed to different electromagnetic frequencies on potato dextrose agar (PDA) Medium along 5 days of incubation at 23 – 25 °C.

Frequency in Hertz (Hz)	Growth diameter (cm) after (days):					*sclerotia
	1	2	3	4	5	
**Control	0.7 ± 0.1	1.6± 0	3.7 ± 0.2	7.3± 0.1	9.0 ± 0	203± 6
1Hz	0.6± 0	1.5 ± 0.1	3.7±0	7.3± 0	9.0 ± 0	199± 10
5Hz	0.6± 0	1.5± 0	3.7± 0.1	7.3± 0.2	9.0± 0	205± 2
10Hz	0.6± 0.1	1.4± 0.2	3.4± 0.2	7.0± 0.1	9.0±0	193± 7
15Hz	0.7± 0.1	1.6± 0.1	4.0± 0.2	7.5± 0	9.0 ± 0	200 ± 1
20Hz	0.8± 0	1.7± 0.1	5.5± 0.1	8.6± 0.2	9.0± 0	---
25Hz	0.6± 0.1	1.6± 0	3.7± 0.25	7.2± 0.1	9.0 ± 0	189± 9

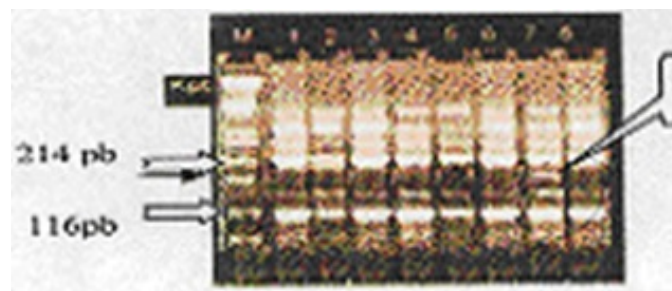
\* Number of Sclerotia produced after 10 days of fungal culture; (Sclerotia which did not form completely are discarded.); \*\* Control means isolates are without any exposure.

**Discussion:**

The data presented in this work indicated that exposure of *Sclerotium* to 20.0 Hz amplitude modulated waves (AMW) resulted in the stop of sclerotia production by the organism. DNA molecular structure study of the irradiated *sclerotium* indicated changes in the structure, as compared with control (unirradiated), which was indicated by the detection of a new band at 190 pb by the primer OPA03. For the analysis of the data one may presume that the resonance interaction of the AMW at 20.0 Hz with the DNA macromolecules of the organism resulted in the interference of these waves with the bioelectric impulses generated in the DNA during metabolic processes which have periodical movements of the micro molecules within the DNA at 50 ms (1/f resonance). This bioelectric resonance interference resulted in the deterioration and/or alteration of the DNA genetic properties which control sclerotia production. However, for more detailed analysis of the data further experimental investigations are needed to be done for the DNA.



**Fig. 3:** PCR products using primers (1-4) for isolates of *Sclerotium cepivorum* isolates (2,4,6 and 8) were exposed to electro magnetic waves with the same frequency (20Hz) isolates (13,5 and 7) are control (without exposure). M=1kb DNA Ladder (DNA Marker)  
Primer 1 = OPD05 (AGGGGTCTTG)  
Primer 2 = OPD05 (AGTCAGCCAC)  
Primer 3 = ITS3 (GCTGCGTTCTTCATCGATGC)  
Primer 1 = ITS2 (GCATCGATGAAGAACGCAGC)



**Fig. 4:** PCR products using primers of OPA03 (AGTCAGCCAC) for isolates of *Sclerotium cepivorum* isolates (2-8) were exposed to electro magnetic waves with frequency 1,5,10,15,20 and 25 respectively and isolates 1is control (without exposure). M=1kb DNA Ladder (DNA Marker).

**Table 2:** Growth diameter of mycelium and sclerotia production of *Sclerotium cepivorum* Isolates exposed to different electromagnetic frequencies on potato dextrose agar (PDA) Medium along 5 days of incubation at 23– 25°C.

Frequency in Hertz (Hz)	Growth diameter (cm ) after (days):					*sclerotia
	1	2	3	4	5	
**Control	0.7± 0	1.6± 0.1	3.9± 0.15	7.4± 0.3	9.0± 0	205± 4
21Hz	0.6± .05	1.5± 0.2	4.1± 0	7.7± 0.3	9.0± 0	190± 5
19Hz	0.6± 0.1	1.5± 0.15	4.0± 0.1	7.5± 0.4	9.0± 0	207± 3
20.5Hz	0.7± 0	1.6± 0.1	4.0± 0	8.1±0.2	9.0± 0	187± 10
19.8Hz	0.7± 0	1.7± 0	4.1± 0.1	8.3± 0	9.0± 0	195± 3
20.2Hz	0.8± 0.1	1.7± 0	5.5± 0	8.5± 0.1	9.0± 0	181± 7
20.3Hz	0.6± 0.1	1.6± 0.1	4.3± 0.2	7.9± 0.2	9.0± 0	180± 2
20Hz	0.8± 0	1.7± 0.1	5.5± 0.1	8.6± 0.2	9.0± 0	-----

\* Number of Sclerotia produced after 10 days of fungal culture; \*\* Control means isolates are without any exposure

**Table 3:** Growth diameter of mycelium and sclerotia production of *Sclerotium cepivorum* Isolates - exposed to 20 Hertz (Hz) electromagnetic frequency - on potato dextrose agar (PDA) Medium along 5 days of incubation at 23- 25°C.

Growth diameter (cm) after (days)	Exposure time to 20 Hertz (Hz) for :				***Control
	1day		3days		
	2hs	4hs	2hs	4hs	
1	0.8± 0	0.7 ± 0.1	0.8 ± 0.1	0.7± 0	0.7± 0
2	1.7± 0	1.7± 0.1	1.7 ± 0.2	1.7± 0	1.7± 0
3	5.5 ± 0.1	5.4 ± 0.1	5.5± 0	5. 3 ± 0.1	4 ± 0.1
4	8.6± 0	8.4 ± 0.15	8.6 ± 0.1	8.65 ± 0.05	7.5 ± 0.1
5	9.0± 0	9.0± 0	9.0± 0	9.0± 0	9.0± 0
*Sclerotia	-----	-----	-----	-----	203± 3

\* Number of Sclerotia produced after 10 days of fungal culture; \*\*\* Control means isolates are without any exposure.

### Conclusion:

The present findings are interesting. It indicates that exposures of the sclerotium to 20.0 Hz AMW for a period of 2 hs. stopped the capability of the organism for sclerotia production. This can be managed in practical use in the field site through the transmission of the resonance wave by an antenna and a power AMW generator to cover some square kilometers areas (depending on the transmitted power to have Vpp of the modulated wave larger than 2V/cm at the treated sclerotia). This new technique has the advantage over any used technique of being efficient in controlling sclerotia production, nontoxic, non-expensive, easy to be applied for large areas and can penetrate land depths up to 40 cm. Annual exposure of the cultured land for only 2 hs will remove sclerotia gradually from infected areas.

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