Effects of long-term exposure to RF/MW radiations on the expression of mRNA of stress proteins in *Lycospersicon esculentum.*

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Abstract: - Low levels of RF radiation exposure can modify the protein's activity by stimulating or inhibiting their expression in cells. The proteinase inhibitor (Pin II) and *Lycospersicon esculentum basic leucine Zipper1* (lebZIP1) are two wound-plants genes. The aim of this work is to study the rate of accumulation of pin II and lebZIP1 at the level of messenger RNA after 10 days of electromagnetic waves exposure. Using RT-PCR and RT-qPCR, the results show that Pin II and lebZIP1synthesis change at the level of cDNA. PinII and lebZIP1 surexpression influence the growth and the differentiation and evoke an increase of protein accumulation in the cell.

Key words: - RF radiation, *Lycospersicon esculentum*, low level exposure; non-thermal effect, Real-Time quantitative PCR.

1 Introduction

The rapid growth of telecommunications technology has raised great concerns about the possible harmful effects of excessive exposure to RF/MW radiations on health. Such studies are of great importance since previous studies showed that the charged ions, molecules and structures inside the body contribute to electromagnetic processes that are crucial for the normal functioning of all living organisms (1).

The effects can be divided into thermal and non-thermal. Certainly the most famous RF interaction mechanism is tissue heating. Thermal effects occur when increasing the temperature by more than 1 °C causing changes at the cellular and molecular levels (2). The amount of heat generated depends on the intensity of the radiation, the electrical properties of the exposed tissue and the body-temperature control mechanisms (3).

However, during the exposure to low intensity radiation, thermal homeostasis remains stable due to the process of thermoregulation. The body temperature can rise up to 1 °C without accumulation. On the basis of this statement, basic exposure limits for the general population have been established and are known as exposure standards (4). Therefore, the problem of prolonged exposure to low-intensity radiation and the non-thermal biological effects of RF/MW remain open to discussion.

A number of published papers treat various modifications and biological effects caused by RF/MW radiation. Conflicting results are not surprising given the variety of factors that affect the experimental procedure including exposure time, exposure to continuous or pulsed waves, polarization, biological systems exposed etc.

Plants are natural receptors for RF/MW since they have structures dedicated to the absorption of light rays (antennas collector for photosynthesis). Previous studies have focused on studying the impact of RF/MW on plants. This approach is justified since the plants are very sensitive to magnetic fields that influence their development (5). Results showed that RF/MW (27 MHz) can alter the rate of movement of the sheets of D. gyrans the telegraph plant (6). Moreover, the physiology of pine (Resin production and aging of needles) could be influenced by RF/MW issued by a radio antenna placed near (7). Other studies showed that flax plants expressed a typical stress response (production of meristems hypocotylaires) after exposure to RF/MW of a mobile phone (8). Although these studies do not control all

of electromagnetic waves exposure conditions, they agree on the fact that plants are influenced by RF/MW radiations and illustrate the interest of using plants to study the effects of the electromagnetic fields (EMF) on living organisms. It is possible to measure in these organisms still universal cellular parameters in controlled conditions. However, the variables described in literature are still few.

The physiological impact of non-ionizing radiation has long been considered as "negligible". Vian et coll 2006 (9) showed that radiation caused a significant overexpression of the transcription factor, similar to that in case of injury caused to the plant.

A herbivore shock or a pathogen attack induces variety of responses including mainly the activation of genes involved in healing and self-defense. The process of wound healing involves a variety of genes involved in cell cycle and differentiation, while the tamper is provided by the expression of genes encoding proteins of pathogen responses (PR) and inhibitors protease (Pin). It seems that the protease inhibitors protect plants against insects and herbivores by reducing the digestibility and nutritional value of leaf protein (10).

The aim of this work is to determine whether the exposure to (EMF) of frequency 1250 MHz can modify the expression of stress proteins in tomato plant and influence their biological function. We are interested in two proteins: the proteinase inhibitor (PinII) and the *Lycospersicon esculentum basic leucine Zipper1* (lebZIP1). This allows the suggestion that this type of radiation is perceived by plants as an environmental stimulus.

2 Materials and methods

2. 1 Plant culture

Seeds of *L. esculentum* have been sown (?) in 6 pots, and then chambered for 3 weeks (until the fourth terminal leaf appears). 3 of them were exposed to the radiofrequency/microwave radiation (1250 MHz) and the other 3 pots were placed in another room without exposure to radiation. After 10 days, leaves were collected and left in air at room temperature for two weeks to dry well. Following that, they were crushed up and grounded by a grinder to get homogeneous fine powder then kept in a dark place at room temperature for further usages in different studies.

2. 2Micro-wave exposure

Microwave exposure of plants has been made in order to create real conditions of low level and permanent electromagnetic field.

We used a microwave source of frequency 1250 MHz and patch antennas to illuminate the plants.

The power has been regulated to create a field of 6V/m and the exposure was carried out for 10 days permanently.

2. 3 Bradford's method

Bradford method was used to monitor the concentration of total protein (11) and to show the change of total protein concentration of exposed cells compared to non-exposed control cells.

2. 4 mRNA extraction

Using Tri-reagent (Sigma), RNA was isolated from frozen tissues.

We homogenized 2 mg of dried leaf with 1mL of TRIR; the homogenate separates into 2 phases after extraction with 0.2mL of chloroform, mixed vigorously, incubated in ice for 5 min, and centrifuged at 12000g for 15 min (4°C).The total RNA was precipitated from aqueous phase by the addition of 0.8mL of isopropanol. Following that incubation for 2 hours at -20°C and centrifugation at 12000g, 15 min, and 4°C took place.

The pellet of RNA was washed with 1mL of ethanol 75% and then solubilized, after drying at 75°C for 3min, with DEPC-water.

2. 5 cDNA synthesis

RT-for-PCR kit (BD Biosciences) was used for cDNA synthesis

We mixed the following components in a 0.2mL Eppendorf tube: 10µl RNA template (less than 1µg); 5µl of one step buffer 10× (15mM of MgCl2); 1 µl of actins primer sense (10µM); 1µl actins primer antisense (10µM); 4µl dNTPs (2.5mM); 1µl RTase (stork is 5 U/µl diluted 1 in 4) 1µl (1.25U) Taq polymerase and the volume was adjusted to 50µl with sterile water. The samples were placed in a thermal cycler and cycled as follows: first strand synthesis (47°C, 30min); RTase blend inactivation and initial denaturation (94°C, 2min/cycle); denaturation (94°C, 20sec); annealing (56°C, 30-40cycles); extension (72°C, 1min/Kb); final extension (72°C,10min/cycle).

The RT- PCR and RT-qPCR primers used for the amplification of *LebZIP1* and were as follow:

The chosen primers

Sense:

5'-GGGATGGAGAAGTTTGGTGGTGG-3', Anti-sense:

5'-CTTCGACCAAGGGATGGTGTAGC-3' amplify just *LebZIP1* cDNA (13) (Genbank accession number *AF176641*)

The RT- PCR and RT-qPCR primers used for the amplification of *PinII* and were as follow:

The chosen primers

Sense:

5'GCCTATTCAAGATGTCCCCGTTCAC3', Anti-sense:

5'CCTTGGGTTCATCACTCTCTCCTTC3' amplify just: *PinII* cDNA(12) (Genbank accession number AY129402)

The RT- PCR and RT-qPCR primers used for the amplification of *Actin* and were as follow:

The chosen primers

Sense:
5'GGGATGGAGAAGTTTGGTGGTGG3'
Anti-sense:
5'CTTCGACCAAGGGATGGTGTAGC3' amplify just: actin cDNA (Genbank accession number BM956640)
A semi-quantitative analysis of RNA expression by RT-PCR, was used to compare the intensity of the bands corresponding to the exposed and control plants knowing that the same volume of cDNA was used for each (5µl). DNA and RNA concentrations were determined by densitometry. Ultraviolet light was used to visualize the Ethidium bromide staining of the PinII and lebZIP1 bands. Quantification of the bands was performed using the supplied software and loading was normalized by the intensity of the Ethidium bromide staining of the DNA ladder bands

2. 6 Real-time quantitative PCR (RT-qPCR) analysis:
Amplifications were conducted on an iCycler iQ (Bio-Rad) with the qPCR Mastermix Plus for SYBR Green I (Eurogentec). The abundance of targeted genes transcripts was normalized to actin mRNA and set relative to the control plant (C, not exposed, harvested before electromagnetic exposure) according to the 2-ΔΔCT method (Livak and Schmittgen, 2001).

3 Results and discussion

3. 1 Germination and growth of seeds
The obtained results showed that after 10 days of exposing this plant to radiofrequency/microwave radiation (1250 MHz), the stems were long and contain less leaves than the control plant. However, after 20 days of exposure to the same frequency the stems were long and contain more leaves than the non-exposed ones. The leaves were larger and thicker in comparison with those of the control plant.

3. 2 Protein accumulation
Bradford's dosage and quantification of the bands after SDS PAGE (result non shown) evidence the increase of 1.87 in total protein level following exposure to continuous wave of RF/MW 1250 MHZ for a period of 10 days. This indicates that indeed the EMF (6 Volt/m), in the absence of thermal effect, induced protein's accumulation either by mRNA surexpression or by inhibiting protein degradation. Then RNA extraction and quantification was performed.

3. 3 RNA Extraction
RNA was isolated from various tissues of control plants or plants exposed to CEM for 10 days. The purified RNA was analyzed by spectrometry; the ratio (OD 260/DO 280) is greater than 2 in both cases. The optical density was 1.6 in control plant and 1.8 in the plant exposed to RF/MW.

3. 4 RT-PCR
The effects of electromagnetic exposure on the rate of the synthesis of mRNA of Pin II and lebZIP1 were studied in agarose gel, after reverse transcriptase reaction and PCR synthesis. The result is illustrated in Fig.1. This study shows a higher expression of mRNA in exposed plants illustrated by an increased RT-PCR products.

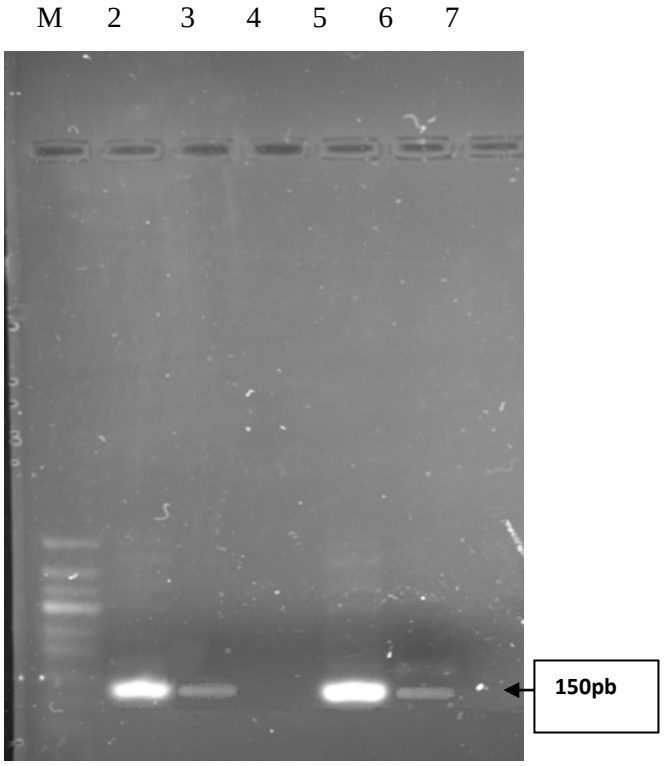

Fig.1 Semi-quantitative analysis of RNA expression by RT-PCR. The RT-PCR products obtained by using primers specific to PinII gene bLepZIP. PinII RT-PCR product (Lane 2, 3) and LebZIP RT-PCR product (Lane 5, 6) exposed (2 ; 5) and control (3 ; 6). . Lane (4 ; 7) negative control .
Lane M, 100bp DNA ladder.

The two products were compared and examined by spectrophotometric method: $OD_{\lambda 260nm}$. = f (concentration of DNA). After quantifying RT-PCR products and the bands using the supplied software, it was found out that plants exposed to EMF showed a significant change in the level of intracellular Pin II and LebZIP1 mRNA synthesis compared to plants cultured without field exposure. The change of pin II and LebZIP1 RT-PCR products exposed 10 days to MF clearly show that exposed plants increased the transcription level of the genes Pin II and LebZIP1. Using RT-PCR it was reported that the expression of mRNA was elevated following exposure to 1250 MHz in the absence of any heating or thermal effect.

3. 5 Real time quantative-PCR:

Exposure of the plant to RF/MW was studied by Real-Time quantitative PCR. The results (see Fig 2A, 2B) show that RF/MW exposure induced 3.2-fold accumulation of mRNA of LebZIP1 related to stress and 2 times the accumulation of the mRNA of PinII. The work done by Vian et coll 2006 (9) showed that the accumulation began just after the end of stimulation and in all cases, the response was maximal 5-15 min after the end of stimulation. In some cases, it decreased slightly after 30 min, but keeping a high- level up to 60 min. In our case, long-term exposures caused high level accumulations that remained for 10 days. When plants were not exposed, no significant accumulation of LebZIP1 and PinII mRNA was observed (Fig. 2, gray). These results indicate that the cellular responses are directly related to the plants' exposure to RF/MW, and the latter is sufficient to induce the PinII and *LebZIP1* mRNA accumulation.

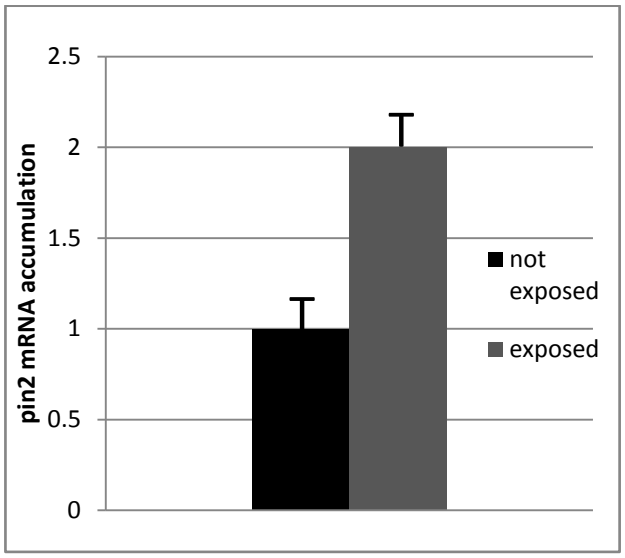

Fig. 2A. Accumulation of *PinII* transcripts after RF/MW exposure. Exposed plants (white bars), control plants (black bars). Each value is expressed relative to the control control (C) and normalized to the actin mRNA and is the average of at least 3 independent repetitions ± the standard error.

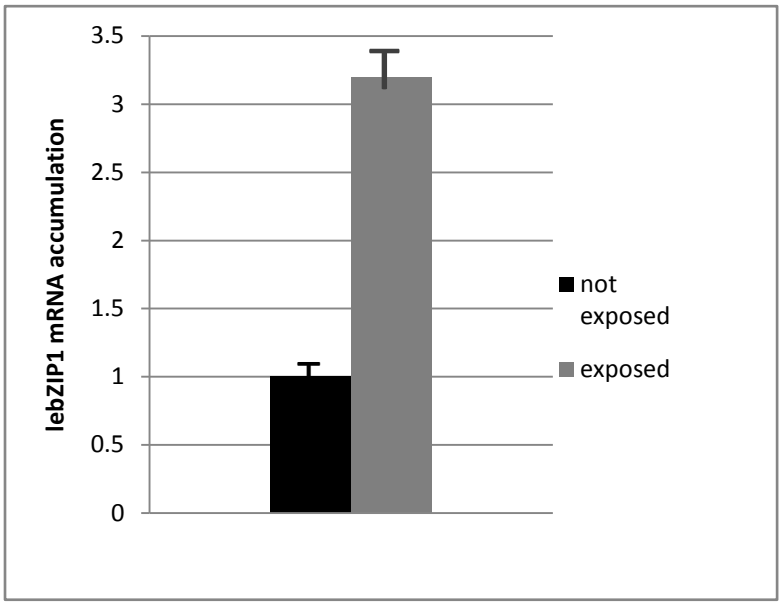

Fig 2B. Accumulation of *LebZIP1* transcripts after RF/MW exposure. Exposed plants (white bars) control plants (black bars). Each value is expressed relative to the control control (C) and normalized to the actin mRNA and is the average of at least 3 independent repetitions ± the standard error.

In addition, the treated tissue has no apparent damage; and the amplitudes of the response are similar to those observed after a strong stimulation as if the plant is ignited.

These results are quite surprising. The mechanism of interaction between the plant and the RF/MW remains a subject of discussion. The energy associated with the radiation RF/MW (16) is extremely low and insufficient to raise the defense mechanisms of plants involving the genesis of free radicals or ionization of molecules. Changes in cytosolic Ca^{2+} concentrations could be the initial signal that triggers the molecular responses observed (14).

The major points emerging from this study is that RF/MW radiations cause an increase in the expression of at least two wound-plants genes. This response is reproducible which allows us to make a formal link between the stimulation of RF/MW and the accumulation of the LebZIP1 mRNA and PinII mRNA.

4 Conclusion

This study highlights the existence of the interaction between the environment and the plants and demonstrates that the genes pinII and LebZIP1 are involved in plant development in response to environmental factors. This fact considering that these genes can be used as tools to improve agronomic crops. For example, the overexpression of LebZIP1 accelerates the growth rate, while overexpressing Pin II produces plants resistant to insect attack. Obviously, further studies are needed to assess whether these genes really give these characteristics to agronomic species. The identification of these genes, followed by the structural and functional characterization, confirms the first step in the biotechnology strategy. At the same time, it is necessary to isolate and characterize suitable inducible promoters and combine them with the necessary genes to induce their expression under the effect of low RF/MW, which represents a very promising task the development and improved varieties of plants.

REFERENCES

[1]. Hyland GJ., Physics and biology of mobile telephony, 356:1833-6, *Lancet* 2000.

[2]. Vecchia P, Matthes R, Ziegelberger G, Lin J, Saunders R, Swerdlow A, editors. [Exposure to High Frequency Electromagnetic Fields, Biological Effects and Health Consequences (100 kHz-300 GHz). *Oberschleisheim: ICNIRP*; 2009.

[3]. International Commission on Non-Ionizing Radiation Protection (ICNIRP). Guidelines for limiting exposure to time-varying electric, magnetic, and electromagnetic fi elds (up to 300 GHz). *Health Phys* 1998;97:257-8.

[4]. World Health Organization (WHO). Electromagnetic Fields (300 Hz to 300 GHz). Environmental Health Criteria 137. *Geneva: WHO*; 1993.

[5]. Galland P, Pazur A. Magnetoreception in plants. *J. Plant. Res*. (2005)118:371–389.

[6]. Ellingsrud S, Johnsson. A Perturbations of plant leaflet rhythms caused by electromagnetic radio-frequency radiation. *Bioelectromagnetics.* (1993) 14(3):257-271.

[7]. Selga T, Selga M. Response of Pinus sylvestris L. needles to electromagnetic fields. Cytological and ultrastructural aspects. *Science of the Total Environment*. (1996). 180(1):65-73.

[8]. Tafforeau M, Verdus MC, Norris V, White G, Demarty M, Thellier M, Ripoll C SIMS study of the calcium-deprivation step related to epidermal meristem production induced in flax by cold shock or radiation from a GSM telephone. *Journal of Trace and Microprobe Techniques*. (2002) 20:611-23.

[9]. Vian A, Roux D, Girard S, Bonnet P, Paladian F, Davies E, Ledoigt G Microwave irradiation affects gene expression in plants. *Plant Signaling & Behavior*. (2006) 1:67-70.

[10]. Bowles DJ. Signal transduction in the wound response of tomato plants. Philosophical *Transactions of the Royal Society. Series B, Biological sciences*. (1998) 353(1374):1495-510.

[11]. Bradford, M B. *Anal. Biochem*. (1976) 72, 248-254.

[12]. Zhang HY, Xie XZ, Xu YZ, Wu NH Isolation and functional assessment of a tomato proteinase inhibitor II gene. *Plant Physiology and Biochemistry*. (2004) 42:437–444.

[13]. Stankovic B, Vian A, Henry-Vian C, Davies E. Molecular cloning and characterization of a tomato cDNA encoding a systemically wound-inducible bZIP DNABinding protein. *Planta*. (2000) 212:60-66.

[14]. Coulon C, Blanchard JM (2001) Flux calciques et expression genique. Medecine Sciences. 17:969-78.

[15]. Livak KJ, Schmittgen TD Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCT Method. *Methods*. (2001) 25, 402-408.

[16]. Challis LJ. Mechanisms for interaction between RF fields and biological tissues. *Bioelectromagnetics supp* 2005; 26:S98-106.