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Non thermal 2.45 GHz electromagnetic exposure causes rapid changes in Arabidopsis thaliana metabolism

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ABSTRACT

Numerous studies report different types of responses following exposure of plants to high frequency electromagnetic fields (HF-EMF). While this phenomenon is related to tissue heating in animals, the situation is much less straightforward in plants where metabolic changes seem to occur without tissue temperature increase. We have set up an exposure system allowing reliable measurements of tissue heating (using a reflectometric probe and thermal imaging) after a long exposure (30 min) to an electromagnetic field of 2.45 GHz transmitted through a horn antenna (about 100 V m^{-1} at the plant level). We did not observe any heating of the tissues, but we detected rapid increases (60 min) in the accumulation of transcripts of stress-related genes (*TCH1* and *ZAT12* transcription factor) or involved in ROS metabolism (*RBOHF* and *APX1*). At the same time, the amounts of hydrogen peroxide and dehydroascorbic acid increased while glutathione (reduced and oxidized forms), ascorbic acid, and lipid peroxidation remained stable. Therefore, our results unambiguously show that molecular and biochemical responses occur rapidly (within 60min) in plants after exposure to an electromagnetic field, in absence of tissue heating.

1. Introduction

In nature, plants are increasingly subjected to various kind of high frequency electromagnetic fields (HF-EMF) due to the densification of base station networks that comply with the exposure limits defined by the standards (ICNIRP, 2020; Israel et al., 2013). The question of whether high-frequency electromagnetic fields are likely to induce changes in plant metabolism and/or development remains however largely open, although many studies tend to show that they do (Kaur et al., 2021; Vian et al., 2016). Biological effects of electromagnetic field are generally interpreted in term of specific absorption rate (SAR) that relies on tissue heating after exposure to HF-EMF. No clear biological changes have been proven to occur in animal or human cells after EMF exposure in the absence of thermal effect (Habauzit et al., 2014; Roux et al., 2010). However, some studies clearly suggested that it could be different in plants in which various kind of biological responses have been detected after exposure to low amplitude, non-thermal EMF. Indeed, Halgamuge et al. (2015) have shown that soybean seedlings exposed to various levels of 900 MHz HF-EMF (SAR of 4.8 10^{-7} to 20 10^{-3} WKg^{-1}) for durations ranging from 2h to 5 days induced changes (mainly growth inhibition) of the plant root and hypocotyl. Similarly, 4h exposure to HF-EMF (1800 MHz, SAR of 1.69 10^{-1} Wkg^{-1}) affected root development, coleoptile length and total chlorophyll content in maize (Kumar et al., 2016). Short exposure of rose buds to low amplitude HF-EMF (900 MHz, SAR of 7.2 10^{-4} W kg^{-1}) in a mode stirred reverberation chamber (MSRC) did not change the length of the axis produced after bud outgrowth, but the post-formed axillary buds produced 45% shorter axis, suggesting that growth changes may occur in a delayed manner (Grémiaux et al., 2016). Recently, Upadhyaya et al. (2022) noted after 12–120h exposure to HF-EMF (1800 MHz, SAR of 3.16 10^{-2} W kg^{-1}) a decrease of seed germination, a reduced growth of plantlets, a reduction of leaf size and an increase in H_2O_2 production. Environmental sensing and signaling in plants are largely dependent on reactive oxygen species (ROS) as signaling molecules, particularly H_2O_2

Abbreviations: HF-EMF, High Frequency-Electromagnetic Field; SAR, Specific Absorption Rate.

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which is the most stable and transportable ROS, involved in subcellular signaling as well as in cell-to-cell signaling (Mittler et al., 2022). Singh et al. (2012) observed reduced growth of mung bean seedlings hypocotyl and roots after 2h low amplitude HF-EMF exposure, along with a 4.5-fold elevation of H_2O_2 content and to concomitant increase of the activities of several antioxidant enzymes: superoxide dismutase (4-fold), catalase (3.3-fold), ascorbate peroxidase (2.5-fold), guaiacol peroxidase (2-folds) and glutathione reductase (5-fold). Similar finding was reported by Chandel et al. (2017) in *Allium cepa* roots after 4h exposure to low level HF-EMF (2100 MHz, SAR of 2.82 10^{-1} W kg^{-1}): H_2O_2 content was increased by 5-fold, while superoxide dismutase and catalase activities were up-regulated 2.4-fold. Gene expression was also affected after exposing plants to low level (non-thermal) HF-EMF: tomato plants exposed to 10 min at 900 MHz (5 V m^{-1}) displayed rapid and transient changes in the expression of several stress-related genes, namely calmodulin, calcium-dependent protein kinase, *bZIP* transcription factor, proteinase inhibitor (*PIN2*) and Chloroplast mRNA Binding Protein (*CMBP*, Roux et al., 2006; Roux et al., 2008; Vian et al., 2006). These results were partially and independently replicated (Rammal et al., 2014), although these authors used a much less sophisticated exposure system and a different exposure condition (10 days, 1250 MHz, 6 V m^{-1}). These changes occurred in a systemic way, since an exposure of a single leaf to HF-EMF triggered changes in gene expression not only locally (*i.e.* at the site where the exposure to HF-EMF was performed), but also at distance in the terminal leaf that was shielded from HF-EMF (Beaubois et al., 2007). More recently, Kundu et al. (2021a) showed that exposure of rice plants over a long period of time (6h per day for 32 days, 2.75 mW m^{-2}) resulted in a significant increase in calmodulin, calcium dependent protein kinase and phytochrome C genes expression. Furthermore, the same research group (Kundu et al., 2021b) demonstrated in 40 days-old 'Swarnaprabha' rice plants that a single HF-EMF irradiation (2 h 30 min, 1837.50 MHz, 2.75 mWm^{-2}) increased the expression level of some stress and light-signaling related genes: calmodulin (2.5-fold), *bZIP* transcription factor (2.27-fold) and phytochrome B and C (3.98- and 5.87-fold, respectively). A mechanism that integrates the rapid responses of plants to exposure to very high frequency electromagnetic waves has recently been proposed (Kaur et al., 2021).

In the present work, we used *Arabidopsis thaliana* as a plant model to assess whether a short (30 min), non-thermal exposure to HF-EMF could trigger rapid changes in plant metabolism. We used a horn antenna that delivers an electromagnetic signal with a fixed angle of incidence and polarization to get similar exposure configuration to those used in the vast majority of the works, in preference to the mode-stirring reverberation chamber that we used in our previous work (Vian et al., 2006; Roux et al., 2006), which allows to create a homogeneous and isotropic electromagnetic environment, but which remains an equipment that is seldomly used for bioelectromagnetic studies. We paid particular attention to ensure that the exposure (30 min, 2450 MHz) did not induce thermal effects in the plants and investigate the possible resulting effects of this non-thermal exposure in terms of rapid (within 1 h) changes in gene expression and biochemical pathways. Using these carefully controlled experimental conditions, we provide evidence that exposure to non-thermal HF-EMF was perceived by plants and induced metabolic changes in the rosette of *Arabidopsis thaliana*.

2. Materials and methods

2.1. Plant material and culture

Arabidopsis (*Arabidopsis thaliana* ecotype Col-0) were grown in controlled environmental conditions in culture chambers (Binder KBW720) under 150–160 µmol m^{-2}. s^{-1} (provided by OSRAM Fluora L18W/77 fluorescent tubes), 21 ± 1 °C at a 16h light period in an adapted substrate for young plants in module trays (TraySubstrat U44-551, Klasmann-Deilmann, Bourgoin Jallieu, France). The plants were watered by sub-irrigation with a nutritive solution and grown for 4 weeks to obtain rosettes with a diameter of 4–5 cm.

2.2. Plant exposure to electromagnetic field and material sampling

The experiments were set-up in a shielded environment (metal-walled room) to avoid possible interaction with the external (environmental) electromagnetic background. A signal synthesizer (SMB100A, Rohde & Schwarz, Munich, Germany) and modulator (AFG3021B, Tektronix, Beaverton, USA) produced a signal, amplified to its nominal amplitude by a high frequency amplifier (ASO104-30/17, Milmega, Ryde, United Kingdom) and frequency modulated by a 100 kHz square signal. Thus, the amplitude of the signal remained constant while the 100 kHz frequency modulation broadens the spectrum of the radiated signal that was emitted as a high-frequency electromagnetic field through a horn antenna (Model 3115, ETS Lindgren, Cedar Park, USA). The antenna was set-up 60 cm above the plants, with an incidence angle of 45° (Fig. 1) and plants were exposed for 30 min in groups of 12, arranged in a rectangular area of about 20 × 30 cm. The resulting EMF amplitudes were measured at the plant level using a tri-axial probe (EP-601, Narda, Cisano sul Neva, Italy) positioned in the center and at each corner of the plants panel and was found to range from 99 to 110 V m^{-1} at the corners and 125 V m^{-1} in the center, Fig. 2A). In these conditions, and given the opening of the horn antenna (24 × 13.5 cm), the far field criterion was 95 cm, thus placing the plants close to this distance (about 85 cm at the center of the plate) in the Fresnel zone (between Dˆ2/(2*wavelength) and 2*Dˆ2/wavelength) to reach the maximum power density value. The spectral characteristics of the EMF (before amplification) was determined by performing a Fast Fourier Transform of the temporal recording of the EMF signal and showed a typical bandwidth at −20 dBm of about 30 MHz, from 2.43 to 2.46 GHz (Fig. 2B). Arabidopsis plants are rosette-shaped (the floral stem being most of the time not yet formed), thus creating a more homogeneous biological system for the interaction with the electromagnetic waves than it would be possible to achieve with other plants displaying axis with several different orientations (Kundu et al., 2022). The impact of EMF on plants in terms of thermal effect was assayed through two separate yet complementary methods. First, we measured the leaf surface temperature using a Luxtron® reflectometric probe (LumaSense Technologies, Santa Clara, USA). The recording of the temperature evolution was started after leaf temperature stabilized and performed every 5 s all along the EMF exposure. Second, we monitor the leaf temperature evolution with a thermal imaging camera (FLIR E40, Teledyne Technologies, Thousand Oaks, USA) with a built-in calibration of temperature scale. The Specific Absorption Rate (SAR, in WKg^{-1}) corresponding to the exposure conditions was determined according to equation (1):

$$\mathrm{SAR} = C \times (dT/dt)_{t\to 0} \quad (1)$$

Where C is the heat capacity ($J^{-1}Kg^{-1}K^{-1}$), T the sample temperature and t the time elapsed since the beginning of the EMF exposure.

Plant leaves were harvested 15, 30 and 60 min after the end of the 30 min exposure, immediately frozen in liquid nitrogen and lately used for biochemical assays and molecular biology experiments. Control (*i.e.* non exposed plants) were harvested at the same points of kinetics (they were manipulated similarly, except that they were not subjected to EMF exposure). Each sample per condition (control and exposed) and per time point was constituted with a pool of fully developed leaves from three plants. The sampling of the material was repeated three times after three independent exposure experiments.

2.3. RNA isolation, cDNA synthesis and gene expression quantification

Frozen materials were ground to a fine powder using Retsch® grinder machine prior RNA isolation using the Nucleospin RNA plant Mini Kit including DNAse treatment (Macherey Nagel, Hoerdt, France),

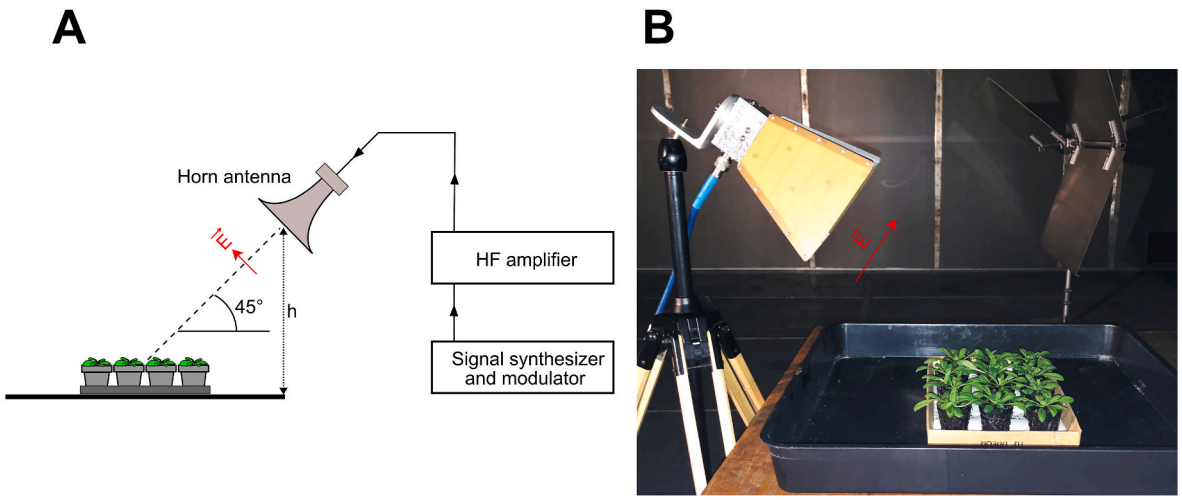


Fig. 1. experimental set-up. The electric polarization is indicated by a red arrow.
A. Schematic representation. The electromagnetic field was generated by a signal synthesizer, amplified, and then emitted through a horn antenna placed at a distance h = 60 cm above the plants with an angle of 45°. **B.** Picture of the set-up. Note that the experiments are performed in a shielded room, totally isolated from the electromagnetic background noise. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

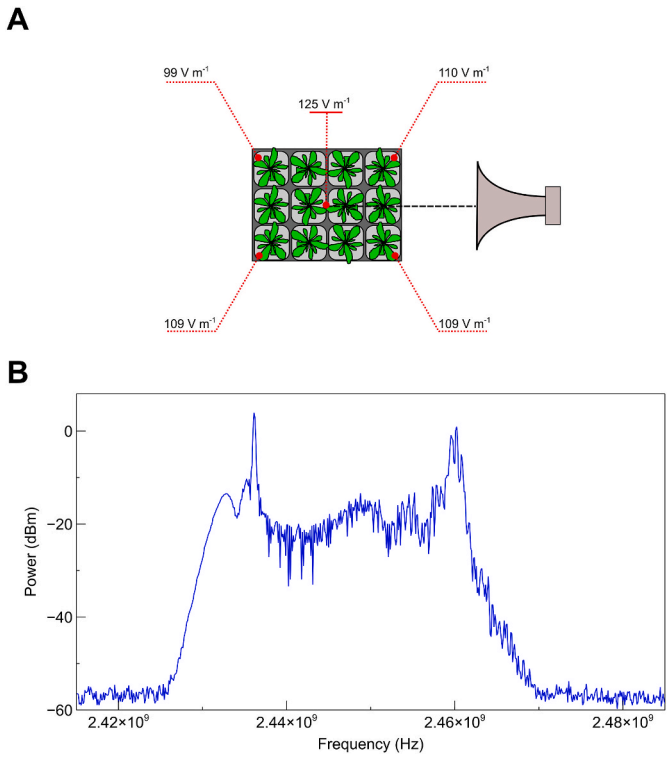


Fig. 2. electromagnetic field characteristics
A. Field amplitude in the plant panel. The field amplitude was measured using a Narda EP-601 triaxial probe in the center and at each corner of the plant panel. **B. Frequency** Bandwidth of the exposure. A Fast Fourier Transform of the temporal signal revealed that the bandwidth of about 30 MHz of the signal is centered on the fundamental frequency (2.45 GHz).

according to the manufacturer instructions. The conversion of mRNAs to cDNA was achieved from 500 ng of total RNA, using the iScript Reverse transcription supermix for RT-qPCR (Bio-Rad, Marnes-la-Coquette, France) following the manufacturer instruction. Relative gene expressions were measured using real time quantitative PCR (RT-qPCR) performed in a final volume of 15 µl of PCR mixture containing 3 µl of 50 times diluted cDNA, 1 µl of primers pairs (10 µM), 4 µl iQ SYBR Green supermix (Bio-Rad, Marnes-la-Coquette, France) and 7 µl of ultrapure water. The amplifications were performed using the CFX96 Real Time System (Bio-Rad) with a standard amplification program previously describe in Porcher et al. (2021). Relative transcript abundances were expressed to the control condition (unexposed samples harvested just after the end of the exposure) after $2^{-\Delta\Delta Ct}$ calculation (Livak and Schmittgen, 2001) along with two house-keeping genes (Aldehyde dehydrogenase, *ALDH* and Glyceraldehyde-3-phosphate dehydrogenase, *GAPDH*), taken as internal references (Jin et al., 2019). The primers sequences used in this study are available in the Supplementary Table 1.

2.4. Hydrogen peroxide quantification

Hydrogen peroxide (H_2O_2) levels were assayed using the Amplex™ Red Hydrogen Peroxide/Peroxidase Assay Kit (Invitrogen). Frozen tissue (20 mg) was homogenized in 100 µl of 1X kit reaction buffer and kept 5 min on ice. After centrifugation (5 min at 14,000×*g* at 4 °C), the supernatant was diluted (10-fold) in 1X reaction buffer. An equal volume of reaction mix freshly prepared according to the manufacturer protocol and mixed to 50 µl of diluted supernatant in a flat bottom black plate (Thermo Scientific™ Nunc™ F96 black MicroWell plate). H_2O_2 levels were determined after a 30 min incubation step (in darkness at room temperature) by fluorescence measurement (570 nm excitation, 590 nm emission) using a microplate reader (BMG Labtech FLUOstar Omega) and a standard curve obtained from known concentrations of commercial H_2O_2 (Sigma).

2.5. Quantification of reduced and oxidized forms of glutathione (GSH/GSSG) and ascorbic acid quantifications (AsA/DHA)

Amounts of the reduced (GSH) and oxidized (GSSG) forms of glutathione were determined from 50 mg of frozen leaf tissues using the GSH-GSSG-Glo™ Assay kit (Promega, Madison, WI, USA) adapted to plant tissues (Porcher et al., 2020). Ascorbic acid (AsA) and dehydroascorbic acid (DHA) quantities were determined from 40 mg of frozen tissues using a colorimetric assay described in Gillespie and Ainsworth (2007).

2.6. Lipid peroxidation assay

Malondialdehyde (MDA) content was assayed as described by Jin et al. (2020). Briefly, 50 mg of finely grinded plant tissue was homogenized in 10% trichloracetic acid and centrifuged for 15 min at 4000×*g*.

The supernatant (300 µl) was added to an equal volume of 0.67% thiobarbituric acid and incubated 30 min at 95 °C under gentle shaking in a dry bath. After a centrifugation (15 min at 10,000×*g*), the supernatant was used to measure absorbances at 450, 532 and 600 nm in a BMG Labtech SpectroStar nano spectrophotometer to determine MDA content (subsequently expressed as µmol.g^{-1}FW) accordingly to equation (2):

$$[\mathrm{MDA}] = 6.45 \times (A_{532} - A_{600}) - 0.56 \times A_{450} \quad (2)$$

2.6.1. Statistical analysis

Statistical analysis was performed with the R statistical software (R Core Team, 2020) using the statistical tests described in figure captions. Graphics were produced using Veusz 3.4 (https://veusz.github.io/) software.

3. Results

3.1. Leaves temperature evolution after HF-EMF exposure and SAR analysis

The 30 min exposure to the 100 kHz frequency modulated 2.45 GHz HF-EMF did not cause any significant increase in leaf temperature, as demonstrated by measurements performed with the Luxtron reflectometric probe (Fig. 3A). The leaves temperature (about 20.3 °C) displayed variations of about 0.1–0.2 °C all along the exposure (30 min) and did not show any significant increase after the application of the EMF exposure (Fig. 3 A, vertical line). Similarly, image acquisition with a thermal imaging camera showed that the surface temperature of the leaves was close to 20 °C before and after exposure to 2.45 GHz EMF, confirming the temperature measurement performed with the Luxtron reflectometric probe and the absence of detectable temperature increase after exposure (Fig. 3 B). However, it cannot be excluded that a slight temperature rise occurred but remained within the temperature measurement uncertainty (0.1 °C). Consequently, we considered a potential maximal leaf temperature increase of 0.1 °C after 30 min of exposure to calculate the corresponding maximum potential SAR value of 0.21 W kg^{-1} according to Eq. (1), assuming a leaf thermal capacity equal to 3.75 kJ K^{-1}kg^{-1} (Kitaya et al., 2003).

3.2. Gene expression after plant exposure to EMF

TCH1 calmodulin transcript remained fairly stable all along the 60 min kinetic in non-exposed plants (Fig. 4A, white bars). In contrast, plant exposure to a non-thermic 100 kHz-modulated 2.45 GHz EMF caused a rapid (15 min) increase in the accumulation of calmodulin transcripts that became significant 60 min after the end of the exposure (Fig. 4A, grey bars). At the same time, the *ZAT12* transcription factor transcript also rapidly accumulated 15 min after the end of EMF and became significantly different from that measured in control, unexposed plants 30 and 60 min after the end of the EMF exposure (Fig. 4B, grey).

We measured the expression level of the NADPH oxidase/respiratory burst oxidase homolog (RBOH), isoforms D and F, that are key enzymes involved in the production of superoxide rapidly dismutates into H_2O_2 by apoplastic superoxide dismutases (SODs), in response to environment constraints (Castro et al., 2021). The expression level of *RBOHD* gene isoform remained essentially constant and unaffected after EMF exposure (Fig. 5A). In contrast, if the accumulation of the *RBOHF* isoform transcript displayed slight changes in the control, unexposed plants (Fig. 5B, white bars), the exposed plants showed constantly higher amounts of transcripts that became statistically significant 60 min after the end of the exposure (Fig. 5B, grey bars), suggesting a higher potential of H_2O_2 production in exposed plants. The expression of the main catalase gene isoform *CAT2* involved in the bulk scavenging of H_2O_2 (Mhamdi et al., 2010), was constant in control plants and not primed by

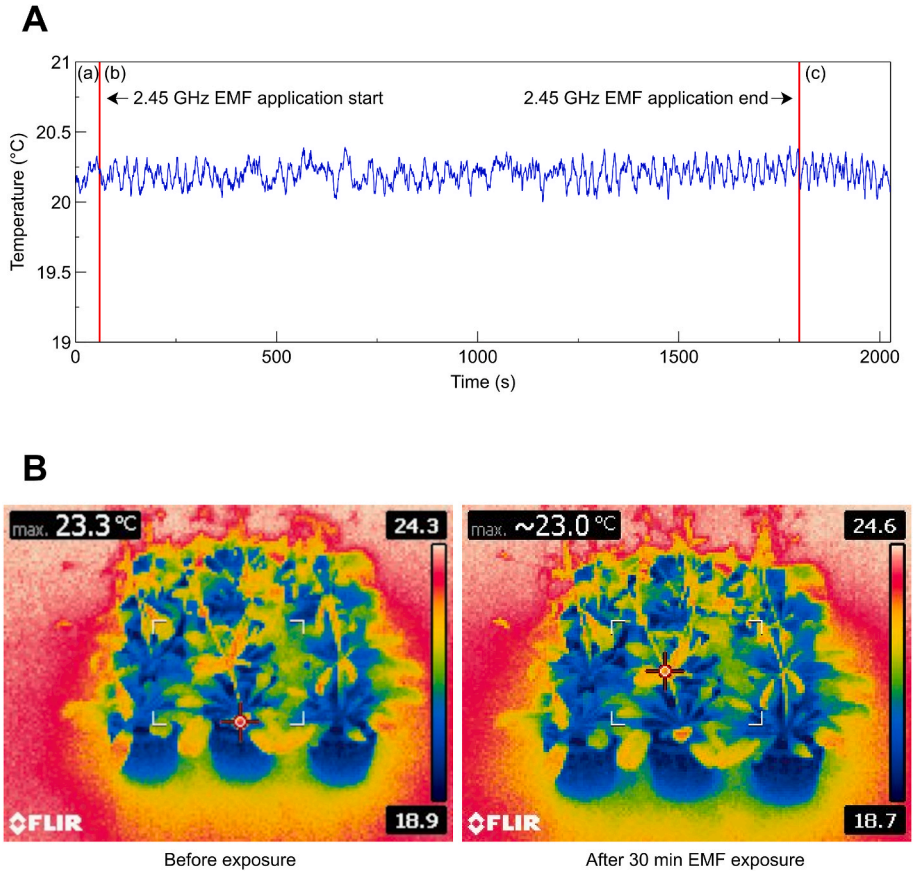


Fig. 3. Evolution of plant leaf temperature after EMF exposure.
A. The leaf surface temperature was continuously recorded from 2 min before the application of the 100 kHz-modulated 2.45 GHz EMF field (a), during the 30 min exposure between the two red vertical lines (b) and 3.5 min after the exposure stop (c). The measurement was performed using a Lumasense Luxtron modèle STS reflectometric probe. Representative recording out of 3 independent experiments. B. Thermic imaging of the plant's panel before (left) of after (right) the application of a 30 min 100 kHz-modulated 2.45 GHz EMF. The red cross materializes the possibility to measure selectively the temperature on a very reduced surface of the leaf. Positioned on hot spots present on both unexposed and exposed samples, the measures revealed similar surface temperatures (24.3 °C and 23 °C, on unexposed and exposed samples, respectively). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

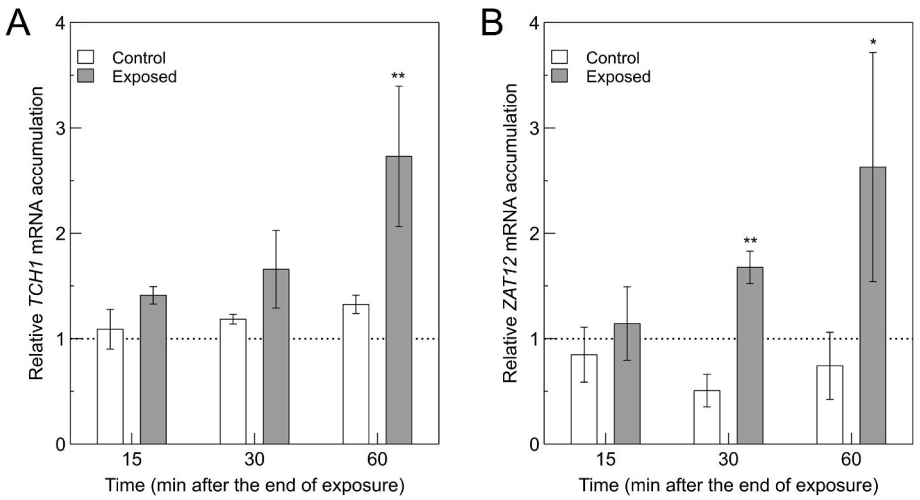


Fig. 4. Effect of HF-EMF exposure on signal transduction-related gene expression

Relative transcript accumulations of *TCH1* calmodulin (**A**) and *ZAT12* transcription factor (**B**) genes, both implicated in rapid responses of plants to various kind of environmental stimuli, in unexposed (control) and exposed plants (15, 30 and 60 min after 30 min exposure to HF-EMF at 2.45 GHz, SAR<0.21 WKg^{-1}). Data are means of n = 3 biological independent replicates ± s. e. Asterisks indicate significant differences after the non-parametric Wilcoxon-Mann-Whitney test at the 95% confidence interval (*: $P < 0.05$; **: $P < 0.01$ and ***: $P < 0.001$) between the exposed and control condition for each different time point.

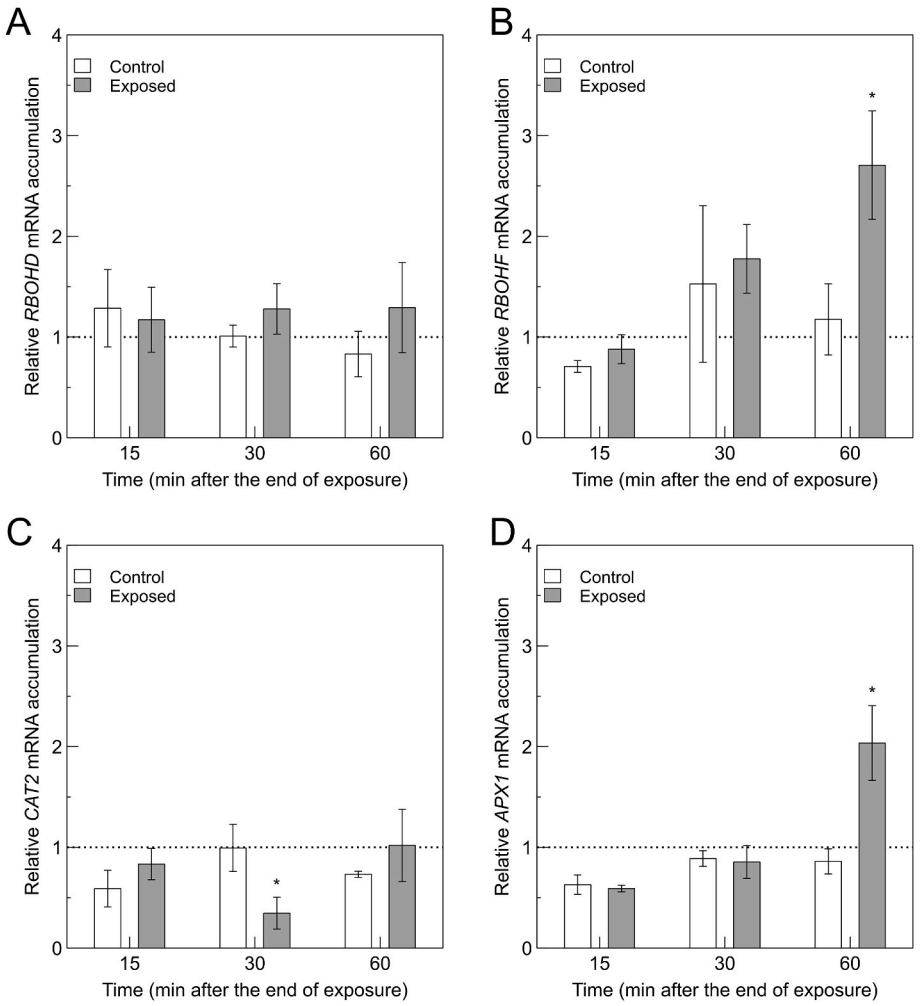


Fig. 5. effect of HF-EMF exposure on ROS-related gene expression

Relative transcript accumulation of (**A**) NADPH oxidases *RBOHD* and (**B**) *RBOHF*, **(C)** catalase *CAT2* and **(D)** ascorbate peroxidase *APX1* genes, all implicated in H_2O_2 production and scavenging, in unexposed (control) and exposed plants (15, 30 and 60 min after 30 min exposure to HF-EMF at 2.45 GHz, SAR<0.21 WKg^{-1}). Data are means of n = 3 biological independent replicates ± s. e. Asterisks indicate significant differences after the non-parametric Wilcoxon-Mann-Whitney test at the 95% confidence interval (*: $P < 0.05$; **: $P < 0.01$ and ***: $P < 0.001$) between the exposed and control condition for each different time point.

EMF exposure (Fig. 5C): a significant decrease in the accumulation of *CAT2* transcript was even noted 30 min after the end of the exposure. In contrast, the expression level of the ascorbate peroxidase *APX1*, involved in the fine tuning of H_2O_2 homeostasis was significantly higher in exposed plants after 60 min (Fig. 5D, grey bars). It is worth noting that it remained essentially constant in control, unexposed plants (Fig. 5D, white bars).

3.3. H_2O_2 accumulation and scavenging

Hydrogen peroxide significantly accumulated 60 min after the end of the exposure, comparatively to that observed in the unexposed control (Fig. 6A). The amount of H_2O_2 was about 213.8 nmol g^{-1} FW while it increased to 293.9 $nmol.g^{-1}FW$ (+37.4%) in the exposed plants.

The amount of total glutathione increase (however not significantly)

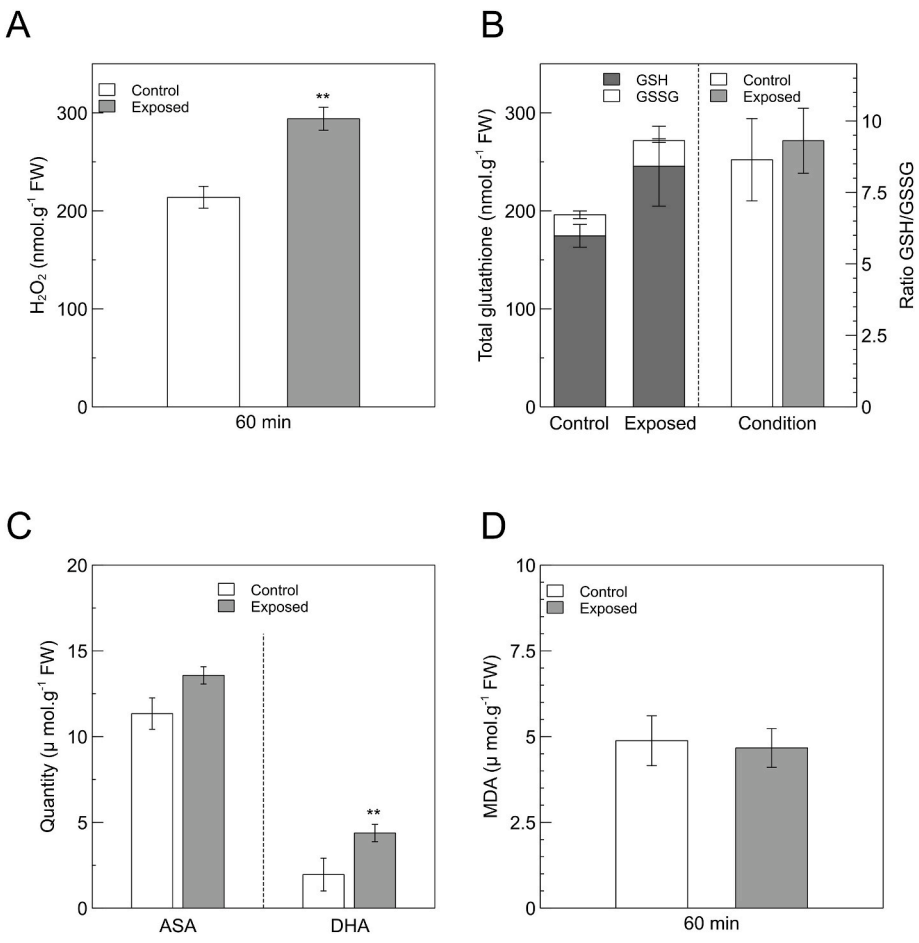


Fig. 6. effect of HF-EMF exposure on ROS metabolism and MDA production
Amounts of (**A**) H_2O_2, (**B**) total glutathione (GSH, GSSG) and GSH/GSSG ratio, (**C**) amounts of ascorbic (ASA) and dehydroascorbic (DHA) acids and (**D**) amounts of malondialdehyde (MDA) in *Arabidopsis thaliana* leaves in unexposed (control) and exposed plants (60 min after 30 min exposure to HF-EMF at 2.45 GHz, SAR<0.21 WKg^{-1}). Data are means of n = 3 biological independent replicates ± s. e. Asterisks indicate significant differences after the non-parametric Wilcoxon-Mann-Whitney test at the 95% confidence interval (*: P < 0.05; **: P < 0.01 and ***: P < 0.001) between the exposed and control condition.

in exposed plants 60 min after the exposure (Fig. 6B, left), mainly due to an elevation in the content of GSH (about 40%, from 174.52 to 245.53 nmol g^{-1} FW in control and exposed plants, respectively), while the amount of GSSG remains approximately the same (21.47 vs 26.11 nmol g^{-1} FW in control and exposed plants, respectively). The amplitude of these changes was not sufficient to significantly shift the GSH/GSSH ratio (8.64 vs 9.31 in control and exposed plants, respectively, Fig. 6B right). The quantification ascorbic acid (ASA) slightly increased (about 19.6%, from 11.34 to 13.57 µmol g^{-1} FW) after exposure, while being not significantly different from that found in unexposed plants (Fig. 6C, right). In contrast, the quantity of dehydroascorbic acid (DHA) was significantly higher (about 124%, from 1.95 to 4.38 µmol g^{-1} FW) 60 min after exposure (Fig. 6C, right).

3.4. Malondialdehyde formation

The amount of malondialdehyde (Fig. 6D) remained very similar in control and exposed plants (4.88 vs 4.67 µmol. g^{-1}FW, respectively) showing that exposing plants for 30 min to a non-thermic 100 kHz-modulated 2.45 GHz EMF did not affect lipid peroxidation.

4. Discussion

The existence of cellular/molecular and morphological changes after non-thermal electromagnetic exposure is largely a matter of debate in the present literature. An EMF-exposure is generally considered as non-thermal if the tissue temperature rise resulting from the exposure is lower than 1 °C after 30 min of exposure (IEEE, 2006; ICNIRP, 2020). Although human cells did not show any significant metabolic changes after such kind of exposure (Roux et al., 2010; Habauzit et al., 2014), the situation is far from being so straightforward in plants. Indeed, several works highlighted that electromagnetic exposures of various frequencies and consistent SAR levels (about 0.5 Wkg^{-1}) caused different kind of cellular responses, including cellular changes and mitotic alteration (Vian et al., 2016; Kaur et al., 2021). Răcuciu et al. (2015) showed that exposing maize seedlings to 1 GHz for 8 h (DAS of 0.47 Wkg^{-1}) resulted in an important decrease in the total pigment (a + b chlorophylls and carotenoids) and a reduction of plantlets growth. Akbal et al. (2012) demonstrated that 48 h exposure of dormant *Lens culinaris* seeds to HF-EMF (1800 MHz, SAR 0.76 W kg^{-1}) did not affect germination but caused a delayed root growth inhibition and an increase in c-mitosis number. Other DNA alterations (increase in micronuclei, ranging from 2.3 to 7-fold compared to non-exposed samples) were found after exposing *Vicia faba* root tips to 915 MHz (SAR: 0.4–1.6 W kg^{-1}) during 72 h (Gustavino et al., 2016). Pesnya and Romanovsky (2013) also observed an increase in mitotic abnormalities after exposing onion root tips to 900 MHz for 9 h (SAR: 1.4 W kg^{-1}). Although it is likely that such alterations are accompanied by changes in gene expression, relatively few studies have investigated this aspect. Engelmann et al. (2008) analyze the global gene expression pattern based on Affymetrix ATH1 microarrays after exposing cultured *Arabidopsis thaliana* cells for 24h to HF-EMF (1.9 GHz, average SAR: 0.75 Wkg^{-1}) and found that 10 genes displayed a significant yet limited variation in their expression (2.5 fold).

Here we used a horn antenna (Fig. 1) to generate an electromagnetic field whose characteristics were deterministic with a fixed incidence, contrary to a finite number of plane waves with random polarization and incidence angle, as in the MSRC that we used in our former studies (Beaubois et al., 2007; Grémiaux et al., 2016; Roux et al., 2008; Vian et al., 2006). This point was essential to ensure that all the waves

contributed to the coupling with the plants (here represented by the rosettes of *Arabidopsis* on which the waves reached with an angle of incidence of 45°, Fig. 1). We used in the present work an exposure condition that keep constant the amplitude of the electromagnetic wave (2.45 GHz, about 100 V m^{-1} at the plant level, Fig. 2A) while the 100 kHz frequency modulation broadens the spectrum of the radiated signal (about 30 MHz bandwidth, Fig. 2B). This exposure condition did not cause any measurable shift in leaf surface temperature over the 30 min of the exposure duration (the temperature oscillation was less than 0.2 °C), as reported by two different measurement methods: the Luxtron reflectometric probe and the thermal imaging (Fig. 3A and B). This absence of tissue heating, that allowed us to selectively study the non-thermal responses of plants to HF-EMF exposure, may be related to the high surface to volume ratio of the plants (Vian et al., 2007) that strongly favor heat dissipation. One could note that the resolution of the thermal camera sensor does not exclude the existence of microscopic temperature rise that are however unlikely to affect the whole leaf temperature.

Taking the uncertainty of the temperature measurement as the maximum potential heating value (here 0.1 °C) resulted in a maximal potential SAR value of 0.21 W kg^{-1}, assuming a leaf thermal capacity equal to 3.75 kJ $K^{-1}kg^{-1}$ (Kitaya et al., 2003). It is worth noting that this potential maximal value was also comparable to those measured in other works reporting biological effects after exposing plants to HF-EMF: 0.169 Wkg^{-1} (Kumar et al., 2016), 0.282 W kg^{-1} (Chandel et al., 2017), for which no temperature increase was neither observed.

We reported here, in this non-thermal exposure condition, that the expression of stress-related genes such as calmodulin *TCH1* (Fig. 4A) or the transcription factor *ZAT12* (Fig. 4B) significantly increased 60 min after the end of the exposure, while remained at a low level in the control, unexposed plants. *TCH1* gene expression was previously reported to increase in tomato after exposure to non-thermal HF-EMF (Roux et al., 2006, 2008), a result later confirmed by Rammal et al. (2014) also in tomato and more recently by Kundu et al. (2021a,b) in rice. The expression level of stress-related transcription factor gene *bZIP* was also reported to increase in such condition (Vian et al., 2006; Beaubois et al., 2007), a result also recently confirmed after exposing rice to low power HF-EMF (Kundu et al., 2021a, 2021b). It is worth noting that the accumulations of TCH1, bZIP and PIN2 occurred more rapidly (0–15 min after the end of the 10 min exposure) when the exposure was performed in a MSRC (Roux et al., 2006, 2008; Beaubois et al., 2007). This difference may result from the multiple reflections of the electromagnetic waves on the metallic walls of the CRBM, which considerably increase the possibilities of coupling of the electromagnetic field with the plant. Changes in the expression levels of calmodulin (*TCH1*) and *ZAT12* genes suggested an activation of calcium and ROS signal transduction pathways that are closely related (Davletova et al., 2005; Gilroy et al., 2016). We therefore analyzed the expression level of several genes corresponding to the main actors of production and scavenging of H_2O_2. We showed that the expression level of the *RBOHD* gene, involved in hypoxia signaling (Yang and Hong, 2015) was not affected after exposing plants to HF-EMF (Fig. 5A). In contrast, *RBOHF*, which appears as a regulation hub targeted by calcium-inducible protein kinases (Han et al., 2019; Castro et al., 2021), was induced after exposure and became significantly more expressed after 60 min (Fig. 5B), suggesting an increase in H_2O_2 production and confirmed by its direct quantification (Fig. 6A). Interestingly, this was concomitant with the increased expression of *TCH1* (Fig. 4A) that is a marker of an increase in cytosolic calcium concentration.

The expression level of catalase (*CAT2*), an essential pathway of bulk, low affinity H_2O_2 detoxification (Gill and Tuteja, 2010), was not affected by HF-EMF exposure (Fig. 5C), whereas that of *APX1*, which finely regulates the H_2O_2 level due to its high affinity, was significantly increased after 60 min (Fig. 5D), indicating a possible involvement of the AsA/glutathione detoxification pathway. The total quantity of glutathione did not change after exposure (Fig. 6B), as well as the GSH/GSSH ratio. Ascorbic acid (Fig. 6C, left) level did not change either, while the dehydroascorbic acid was the only actor of the AsA/glutathione pathway displaying a significant variation 60 min after exposure (Fig. 6C, right). Taken together, these results showed that non-thermic HF-EMF trigger a low but significant increase in H_2O_2 production which reflects more of a signal transduction mechanism, acting in synergy with possible calcium movements as reported by calmodulin increase, than a stress response. This observation is consistent with the general scheme proposed by Kaur et al. (2021) in which the initial interaction of electromagnetic field with plants triggers both calcium movements and ROS metabolism as initial events of signal transduction cascade that later cause changes in plant development. Modifications in ROS production and/or scavenging were in fact reported among others in wheat (Chen et al., 2009), mung bean (Singh et al., 2012), onions (Chandel et al., 2017), duckweed (Tkalec et al., 2007) and Arabidopsis (Senevirathna et al., 2020). It is also striking that quite similar changes in ROS metabolism have been reported after exposure of plants to a static magnetic field (SMF) or to an extremely low frequency electromagnetic field (ELF-EMF), despite the fundamental differences in the nature of these fields. Indeed, exposing plants to static magnetic field higher than the geomagnetic field generally caused an increase in H_2O_2 production and ROS-scavenging enzymes activities and H_2O_2 synthesis-related gene expression (see the reviews from Maffei (2014)), Saletnik et al. (2022) and Hafeez et al. (2023) for details). In contrast, exposing plants to near null magnetic field (much lower to that of the geomagnetic field) resulted in a drastic reduction of H_2O_2 in roots and shoots (5-folds after 24h), likely related to the up-regulation of gene expression of ROS scavenging enzymes (Parmagnani et al., 2022). ROS metabolism is therefore an essential component in plants' perception of the presence/absence of static magnetic fields, as well as of their exposure to very high frequency electromagnetic fields.

5. Conclusion

We demonstrated here that a very high frequency electromagnetic field, which we have verified as having no heating effects in plants, was able to induce rapid changes in the expression of several genes involved in signal transduction or ROS metabolism, as well as the production of hydrogen peroxide. This ability appears to be unique to plants, as it is clear that non-thermal exposure does not elicit a molecular response in humans (Roux et al., 2010; Habauzit et al., 2014). These results, in addition to many previous observations, raise the question of the relevance of SAR in plants, insofar as this quantity (which otherwise adequately accounts for biological effects in animals) is based on the thermal effects induced by exposure to electromagnetic fields. This major difference could be the consequence of the fundamental contrast in the logic of development (Vian et al., 2007), as plants create large surfaces, which forces them to immobility but favors interaction with the environment (in this case interaction with electromagnetic waves), whereas animals create volume and comparatively little surface with the environment (which favors their mobility). The interaction mechanism of the electromagnetic field, which clearly does not depend on thermal effects, remains as yet misunderstood although several hypotheses have been proposed (Cifra et al., 2011; Romanenko et al., 2017). Studies are still needed, and we believe that a more comprehensive knowledge of the molecular and biochemical changes (such as those provided by an RNA sequencing or metabolic analyses) taking place after non-thermal exposure could allow us to make effective progress in understanding the mechanism of interaction between plants and electromagnetic field.

CRediT authorship contribution statement

AP, SG, PB, RR, VG, FP and AV conceived the experiments. AP, SG and AV made the experiments. AP and AV write the manuscript draft. SG, PB, RR, VG and FP proof-read the manuscript. All authors have approved the manuscript.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Francoise Paladian reports was provided by SNCF Réseau.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jplph.2023.153999.

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