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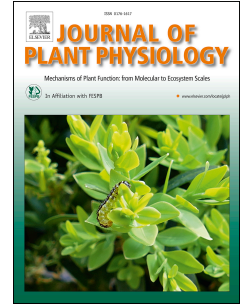


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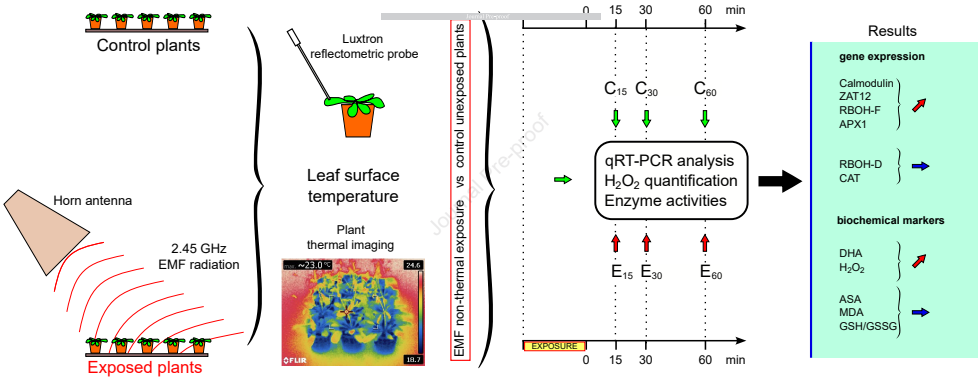
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AP, SG, PB, RR, VG, FP and AV conceived the experiments. AP, SG and AV made the experiments. AP and AV write the manuscript draft. SG, PB, RR, VG and FP proof-read the manuscript. All authors have approved the manuscript.

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1 Title : Non thermal 2.45 GHz electromagnetic exposure causes rapid changes in Arabidopsis
2 thaliana metabolism.

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24 **Abstract**

25

26

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

41

42 **Keywords** : electromagnetic exposure, non-thermal, stress-marker genes, hydrogen
43 peroxide

44

45 **Abbreviations:**

46 HF-EMF: High Frequency-Electromagnetic Field

47 SAR: Specific Absorption Rate

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51 **Introduction**

52 In nature, plants are increasingly subjected to various kind of high frequency electromagnetic
53 fields (HF-EMF) due to the densification of base station networks that comply with the exposure
54 limits defined by the standards (ICNIRP, 2020; Israel et al., 2013). The question of whether
55 high-frequency electromagnetic fields are likely to induce changes in plant metabolism and/or
56 development remains however largely open, although many studies tend to show that they do
57 (Kaur et al., 2021; Vian et al., 2016). Biological effects of electromagnetic field are generally
58 interpreted in term of specific absorption rate (SAR) that relies on tissue heating after exposure
59 to HF-EMF. No clear biological changes have been proven to occur in animal or human cells
60 after EMF exposure in the absence of thermal effect (Habauzit et al., 2014; Roux et al., 2010).
61 However, some studies clearly suggested that it could be different in plants in which various
62 kind of biological responses have been detected after exposure to low amplitude, non-thermal
63 EMF. Indeed, Halgamuge et al. (2015) have shown that soybean seedlings exposed to various
64 levels of 900 MHz HF-EMF (SAR of $4.8 \cdot 10^{-7}$ to $20 \cdot 10^{-3} \text{ Wkg}^{-1}$) for durations ranging from 2h to
65 5 days induced changes (mainly growth inhibition) of the plant root and hypocotyl. Similarly,
66 4h exposure to HF-EMF (1800 MHz, SAR of $1.69 \cdot 10^{-1} \text{ Wkg}^{-1}$) affected root development,
67 coleoptile length and total chlorophyll content in maize (Kumar et al., 2016). Short exposure of
68 rose buds to low amplitude HF-EMF (900 MHz, SAR of $7.2 \cdot 10^{-4} \text{ W kg}^{-1}$) in a mode stirred
69 reverberation chamber (MSRC) did not change the length of the axis produced after bud
70 outgrowth, but the post-formed axillary buds produced 45% shorter axis, suggesting that
71 growth changes may occur in a delayed manner (Grémiaux et al., 2016). Recently, Upadhyaya
72 et al. (2022) noted after 12-120h exposure to HF-EMF (1800 MHz , SAR of $3.16 \cdot 10^{-2} \text{ W kg}^{-1}$)
73 a decrease of seed germination, a reduced growth of plantlets, a reduction of leaf size and an
74 increase in H_2O_2 production. Environmental sensing and signaling in plants are largely
75 dependent on reactive oxygen species (ROS) as signaling molecules, particularly H_2O_2 which
76 is the most stable and transportable ROS, involved in subcellular signaling as well as in cell-
77 to-cell signaling (Mittler et al., 2022). Singh et al. (2012) observed reduced growth of mung
78 bean seedlings hypocotyl and roots after 2h low amplitude HF-EMF exposure, along with a

79 4.5-fold elevation of H₂O₂ content and to concomitant increase of the activities of several
80 antioxidant enzymes: superoxide dismutase (4-fold), catalase (3.3-fold), ascorbate peroxidase
81 (2.5-fold), guaiacol peroxidase (2-folds) and glutathione reductase (5-fold). Similar finding was
82 reported by Chandel et al. (2017) in *Allium cepa* roots after 4h exposure to low level HF-EMF
83 (2100 MHz, SAR of 2.82 10⁻¹ W kg⁻¹): H₂O₂ content was increased by 5-fold, while superoxide
84 dismutase and catalase activities were up-regulated 2.4-fold. Gene expression was also
85 affected after exposing plants to low level (non-thermal) HF-EMF: tomato plants exposed to
86 10 min at 900 MHz (5 Vm⁻¹) displayed rapid and transient changes in the expression of several
87 stress-related genes, namely calmodulin, calcium-dependent protein kinase, *bZIP*
88 transcription factor, proteinase inhibitor (*PIN2*) and Chloroplast mRNA Binding Protein (*CMBP*,
89 Roux et al., 2006; Roux et al., 2008; Vian et al., 2006). These results were partially and
90 independently replicated (Rammal et al., 2014), although these authors used a much less
91 sophisticated exposure system and a different exposure condition (10 days, 1250 MHz, 6 Vm⁻¹
92). These changes occurred in a systemic way, since an exposure of a single leaf to HF-EMF
93 triggered changes in gene expression not only locally (*i.e.* at the site where the exposure to
94 HF-EMF was performed), but also at distance in the terminal leaf that was shielded from HF-
95 EMF (Beaubois et al., 2007). More recently, Kundu et al. (2021a) showed that exposure of rice
96 plants over a long period of time (6h per day for 32 days, 2.75 mW m⁻²) resulted in a significant
97 increase in calmodulin, calcium dependent protein kinase and phytochrome C genes
98 expression. Furthermore, the same research group (Kundu et al., 2021b) demonstrated in
99 40 days-old 'Swarnaprabha' rice plants that a single HF-EMF irradiation (2 h 30 min,
100 1837.50 MHz, 2.75 mWm⁻²) increased the expression level of some stress and light-signaling
101 related genes: calmodulin (2.5-fold), *bZIP* transcription factor (2.27-fold) and phytochrome B
102 and C (3.98- and 5.87-fold, respectively). A mechanism that integrates the rapid responses of
103 plants to exposure to very high frequency electromagnetic waves has recently been proposed
104 (Kaur et al., 2021).

105 In the present work, we used *Arabidopsis thaliana* as a plant model to assess whether a short
106 (30 min), non-thermal exposure to HF-EMF could trigger rapid changes in plant metabolism.
107 We used a horn antenna that delivers an electromagnetic signal with a fixed angle of incidence
108 and polarization to get similar exposure configuration to those used in the vast majority of the
109 works, in preference to the mode-stirring reverberation chamber that we used in our previous
110 work (Vian et al., 2006; Roux et al., 2006), which allows to create a homogeneous and isotropic
111 electromagnetic environment, but which remains an equipment that is seldomly used for
112 bioelectromagnetic studies. We paid particular attention to ensure that the exposure (30 min,
113 2450 MHz) did not induce thermal effects in the plants and investigate the possible resulting
114 effects of this non-thermal exposure in terms of rapid (within one hour) changes in gene
115 expression and biochemical pathways. Using these carefully controlled experimental
116 conditions, we provide evidence that exposure to non-thermal HF-EMF was perceived by
117 plants and induced metabolic changes in the rosette of *Arabidopsis thaliana*.

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133 **Materials and Methods**134 *Plant material and culture*

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

141 *Plant exposure to electromagnetic field and material sampling*

142 The experiments were set-up in a shielded environment (metal-walled room) to avoid possible
143 interaction with the external (environmental) electromagnetic background. A signal synthesizer
144 (SMB100A, Rohde & Schwarz, Munich, Germany) and modulator (AFG3021B, Tektronix,
145 Beaverton, USA) produced a signal, amplified to its nominal amplitude by a high frequency
146 amplifier (ASO104-30/17, Milmega, Ryde, United Kingdom) and frequency modulated by a
147 100 kHz square signal. Thus, the amplitude of the signal remained constant while the 100 kHz
148 frequency modulation broadens the spectrum of the radiated signal that was emitted as a high-
149 frequency electromagnetic field through a horn antenna (Model 3115, ETS Lindgren, Cedar
150 Park, USA). The antenna was set-up 60 cm above the plants, with an incidence angle of 45°
151 (Fig. 1) and plants were exposed for 30 min in groups of 12, arranged in a rectangular area of
152 about 20x30 cm. The resulting EMF amplitudes were measured at the plant level using a tri-
153 axial probe (EP-601, Narda, Cisano sul Neva, Italy) positioned in the center and at each corner
154 of the plants panel and was found to range from 99 to 110 V m^{-1} at the corners and 125 V m^{-1}
155 in the center, Figure 2A). In these conditions, and given the opening of the horn antenna (24 x
156 13.5 cm), the far field criterion was 95 cm, thus placing the plants close to this distance (about
157 85 cm at the center of the plate) in the Fresnel zone (between $D^2/(2*\text{wavelength})$ and

158 $2 \cdot D^2 / \text{wavelength}$) to reach the maximum power value. The spectral characteristics of the
159 EMF (before amplification) was determined by performing a Fast Fourier Transform of the
160 temporal recording of the EMF signal and showed a typical bandwidth at -20 dBm of about
161 30 MHz, from 2.43 to 2.46 GHz (Fig. 2B). Arabidopsis plants are rosette-shaped (the floral
162 stem being most of the time not yet formed), thus creating a more homogeneous biological
163 system for the interaction with the electromagnetic waves than it would be possible to achieve
164 with other plants displaying axis with several different orientations (Kundu et al, 2022). The
165 impact of EMF on plants in terms of thermal effect was assayed through two separate yet
166 complementary methods. First, we measured the leaf surface temperature using a Luxtron®
167 reflectometric probe (LumaSense Technologies, Santa Clara, USA). The recording of the
168 temperature evolution was started after leaf temperature stabilized and performed every 5 s
169 all along the EMF exposure. Second, we monitor the leaf temperature evolution with a thermal
170 imaging camera (FLIR E40, Teledyne Technologies, Thousand Oaks, USA) with a built-in
171 calibration of temperature scale. The Specific Absorption Rate (SAR, in WKg^{-1}) corresponding
172 to the exposure conditions was determined according to equation (1) :

$$173 \quad \text{SAR} = C \times (dT/dt)_{t \rightarrow 0} \quad (1)$$

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

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

183 *RNA isolation, cDNA synthesis and gene expression quantification*

184 Frozen materials were ground to a fine powder using Retsch® grinder machine prior RNA
185 isolation using the Nucleospin RNA plant Mini Kit including DNase treatment (Macherey Nagel,
186 Hoerd, France), according to the manufacturer instructions. The conversion of mRNAs to
187 cDNA was achieved from 500 ng of total RNA, using the iScript Reverse transcription supermix
188 for RT-qPCR (Bio-Rad, Marnes-la-Coquette, France) following the manufacturer instruction.
189 Relative gene expressions were measured using real time quantitative PCR (RT-qPCR)
190 performed in a final volume of 15 µl of PCR mixture containing 3 µl of 50 times diluted cDNA,
191 1 µl of primers pairs (10 µM), 4 µl iQ SYBR Green supermix (Bio-Rad, Marnes-la-Coquette,
192 France) and 7 µl of ultrapure water. The amplifications were performed using the CFX96 Real
193 Time System (Bio-Rad) with a standard amplification program previously describe in Porcher
194 et al. (2021). Relative transcript abundances were expressed to the control condition
195 (unexposed samples harvested just after the end of the exposure) after $2^{-\Delta\Delta C_t}$ calculation (Livak
196 and Schmittgen, 2001) along with two house-keeping genes (Aldehyde dehydrogenase, *ALDH*
197 and Glyceraldehyde-3-phosphate dehydrogenase, *GAPDH*), taken as internal references (Jin
198 et al., 2019). The primers sequences used in this study are available in the supplementary
199 table 1.

200 *Hydrogen peroxide quantification*

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

211 *Quantification of reduced and oxidized forms of glutathione (GSH/GSSG) and ascorbic acid*
212 *quantifications (AsA/DHA)*

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

218 *Lipid peroxidation assay*

219 Malondialdehyde (MDA) content was assayed as described by Jin et al (2020). Briefly, 50 mg
220 of finely grinded plant tissue was homogenized in 10% trichloroacetic acid and centrifuged for
221 15 min at 4000xg. The supernatant (300 µl) was added to an equal volume of
222 0.67% thiobarbituric acid and incubated 30 min at 95°C under gentle shaking in a dry bath.
223 After a centrifugation (15 min at 10,000xg), the supernatant was used to measure absorbances
224 at 450, 532 and 600 nm in a BMG Labtech SpectroStar nano spectrophotometer to determine
225 MDA content (subsequently expressed as µmol.g⁻¹ FW) accordingly to equation 2:

$$226 \quad [\text{MDA}] = 6.45 \times (A_{532} - A_{600}) - 0.56 \times A_{450} \quad (2)$$

227

228 *Statistical analysis*

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

232 Results

233

234 *Leaves temperature evolution after HF-EMF exposure and SAR analysis*

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

249

250 *Gene expression after plant exposure to EMF*

251 *TCH1* calmodulin transcript remained fairly stable all along the 60 min kinetic in non-exposed
252 plants (Figure 4A, white bars). In contrast, plant exposure to a non-thermic 100 kHz-modulated
253 2.45 GHz EMF caused a rapid (15 min) increase in the accumulation of calmodulin transcripts
254 that became significant 60 min after the end of the exposure (Figure 4A, grey bars). At the
255 same time, the *ZAT12* transcription factor transcript also rapidly accumulated 15 min after the
256 end of EMF and became significantly different from that measured in control, unexposed plants
257 30 and 60 min after the end of the EMF exposure (Figure 4B, grey).

258 We measured the expression level of the NADPH oxidase/respiratory burst oxidase homolog
259 (RBOH), isoforms D and F, that are key enzymes involved in the production of superoxide
260 rapidly dismutates into H_2O_2 by apoplastic superoxide dismutases (SODs), in response to
261 environment constraints (Castro et al., 2021). The expression level of *RBOHD* gene isoform
262 remained essentially constant and unaffected after EMF exposure (Figure 5A). In contrast, if
263 the accumulation of the *RBOHF* isoform transcript displayed slight changes in the control,
264 unexposed plants (Figure 5B, white bars), the exposed plants showed constantly higher
265 amounts of transcripts that became statistically significant 60 min after the end of the exposure
266 (Figure 5B, gray bars), suggesting a higher potential of H_2O_2 production in exposed plants.
267 The expression of the main catalase gene isoform *CAT2* involved in the bulk scavenging of
268 H_2O_2 (Mhamdi et al., 2010), was constant in control plants and not primed by EMF exposure
269 (Figure 5C): a significant decrease in the accumulation of *CAT2* transcript was even noted
270 30 min after the end of the exposure. In contrast, the expression level of the ascorbate
271 peroxidase *APX1*, involved in the fine tuning of H_2O_2 homeostasis was significantly higher in
272 exposed plants after 60 min (Figure 5D, grey bars). It is worth noting that it remained
273 essentially constant in control, unexposed plants (Figure 5D, white bars).

274

275 *H₂O₂ accumulation and scavenging*

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

280 The amount of total glutathione increase (however not significantly) in exposed plants 60 min
281 after the exposure (Figure 6B, left), mainly due to an elevation in the content of GSH (about
282 40%, from 174.52 to $245.53 \text{ nmol g}^{-1} \text{ FW}$ in control and exposed plants, respectively), while
283 the amount of GSSG remains approximately the same (21.47 vs $26.11 \text{ nmol g}^{-1} \text{ FW}$ in control

284 and exposed plants, respectively). The amplitude of these changes was not sufficient to
285 significantly shift the GSH/GSSH ratio (8.64 vs 9.31 in control and exposed plants,
286 respectively, Fig 6B right). The quantification ascorbic acid (ASA) slightly increased (about
287 19.6%, from 11.34 to 13.57 $\mu\text{mol g}^{-1}$ FW) after exposure, while being not significantly different
288 from that found in unexposed plants (Fig 6C, right). In contrast, the quantity of dehydroascorbic
289 acid (DHA) was significantly higher (about 124%, from 1.95 to 4.38 $\mu\text{mol g}^{-1}$ FW) 60 min after
290 exposure (Fig 6C, right).

291

292 *Malondialdehyde formation*

293 The amount of malondialdehyde (Fig 6D) remained very similar in control and exposed plants
294 (4.88 vs 4.67 $\mu\text{mol. g}^{-1}$ FW, respectively) showing that exposing plants for 30 min to a non-
295 thermic 100 kHz-modulated 2.45 GHz EMF did not affect lipid peroxidation.

296

297 **Discussion**

298 The existence of cellular/molecular and morphological changes after non-thermal
299 electromagnetic exposure is largely a matter of debate in the present literature. An EMF-
300 exposure is generally considered as non-thermal if the tissue temperature rise resulting from
301 the exposure is lower than 1°C after 30 min of exposure (IEEE, 2005; ICNIRP, 2020). Although
302 human cells did not show any significant metabolic changes after such kind of exposure (Roux
303 et al., 2010; Habauzit et al., 2014), the situation is far from being so straightforward in plants.
304 Indeed, several works highlighted that electromagnetic exposures of various frequencies and
305 consistent SAR levels (about 0.5 Wkg^{-1}) caused different kind of cellular responses, including
306 cellular changes and mitotic alteration (Vian et al., 2016; Kaur et al., 2021). Răcuciu et al.
307 (2015) showed that exposing maize seedlings to 1 GHz for 8 h (DAS of 0.47 Wkg^{-1}) resulted
308 in an important decrease in the total pigment (a + b chlorophylls and carotenoids) and a
309 reduction of plantlets growth. Akbal et al (2012) demonstrated that 48 h exposure of dormant
310 *Lens culinaris* seeds to HF-EMF (1800 MHz, SAR 0.76 W kg^{-1}) did not affect germination but

311 caused a delayed root growth inhibition and an increase in c-mitosis number. Other DNA
312 alterations (increase in micronuclei, ranging from 2.3 to 7-fold compared to non-exposed
313 samples) were found after exposing *Vicia faba* root tips to 915 MHz (SAR: 0.4-1.6 W kg⁻¹)
314 during 72 h (Gustavino et al., 2016). Pesnya and Romanovsky (2013) also observed an
315 increase in mitotic abnormalities after exposing onion root tips to 900 MHz for 9 h (SAR:
316 1.4 W kg⁻¹). Although it is likely that such alterations are accompanied by changes in gene
317 expression, relatively few studies have investigated this aspect. Engelmann et al. (2008)
318 analyze the global gene expression pattern based on Affymetrix ATH1 microarrays after
319 exposing cultured *Arabidopsis thaliana* cells for 24h to HF-EMF (1.9 GHz, average SAR:
320 0.75 Wkg⁻¹) and found that 10 genes displayed a significant yet limited variation in their
321 expression (2.5 fold).

322 Here we used a horn antenna (Fig. 1) to generate an electromagnetic field whose
323 characteristics were deterministic with a fixed incidence, contrary to a finite number of plane
324 waves with random polarization and incidence angle, as in the MSRC that we used in our
325 former studies (Beaubois et al., 2007; Grémiaux et al., 2016; Roux et al., 2008; Vian et al.,
326 2006). This point was essential to ensure that all the waves contributed to the coupling with
327 the plants (here represented by the rosettes of *Arabidopsis* on which the waves reached with
328 an angle of incidence of 45°, Fig. 1). We used in the present work an exposure condition that
329 keep constant the amplitude of the electromagnetic wave (2.45 GHz, about 100 Vm⁻¹ at the
330 plant level, Fig. 2A) while the 100 kHz frequency modulation broadens the spectrum of the
331 radiated signal (about 30 MHz bandwidth, Fig. 2B). This exposure condition did not cause any
332 measurable shift in leaf surface temperature over the 30 min of the exposure duration (the
333 temperature oscillation was less than 0.2 °C), as reported by two different measurement
334 methods: the Luxtron reflectometric probe and the thermal imaging (Fig. 3A-B). This absence
335 of tissue heating, that allowed us to selectively study the non-thermal responses of plants to
336 HF-EMF exposure, may be related to the high surface to volume ratio of the plants (Vian et al.,
337 2007) that strongly favor heat dissipation. One could note that the resolution of the thermal

338 camera sensor does not exclude the existence of microscopic temperature rise that are
339 however unlikely to affect the whole leaf temperature.

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

346 We reported here, in this non-thermal exposure condition, that the expression of stress-related
347 genes such as calmodulin *TCH1* (Fig. 4A) or the transcription factor *ZAT12* (Fig. 4B)
348 significantly increased 60 min after the end of the exposure, while remained at a low level in
349 the control, unexposed plants. *TCH1* gene expression was previously reported to increase in
350 tomato after exposure to non-thermal HF-EMF (Roux *et al.*, 2006; Roux *et al.*, 2008) (,a result
351 later confirmed by Rammal *et al.* (2014) also in tomato and more recently by Kundu *et al.*
352 (2021a,b) in rice. The expression level of stress-related transcription factor gene *bZIP* was also
353 reported to increase in such condition (Vian *et al.*, 2006; Beaubois *et al.*, 2007), a result also
354 recently confirmed after exposing rice to low power HF-EMF (Kundu *et al.*, 2021a,b). It is worth
355 noting that the accumulations of *TCH1*, *bZIP* and *PIN2* occurred more rapidly (0-15 min after
356 the end of the 10 min exposure) when the exposure was performed in a MSRC (Roux *et al.*,
357 2006, 2008; Beaubois *et al.*, 2007). This difference may result from the multiple reflections of
358 the electromagnetic waves on the metallic walls of the CRBM, which considerably increase the
359 possibilities of coupling of the electromagnetic field with the plant. Changes in the expression
360 levels of calmodulin (*TCH1*) and *ZAT12* genes suggested an activation of calcium and ROS
361 signal transduction pathways that are closely related (Davletova *et al.*, 2005; Gilroy *et al.*,
362 2016). We therefore analyzed the expression level of several genes corresponding to the main
363 actors of production and scavenging of H₂O₂. We showed that the expression level of the
364 *RBOHD* gene, involved in hypoxia signaling (Yang and Hong, 2015) was not affected after

365 exposing plants to HF-EMF (Fig. 5A). In contrast, *RBOHF*, which appears as a regulation hub
366 targeted by calcium-inducible protein kinases (Han et al, 2018; Castro et al., 2021), was
367 induced after exposure and became significantly more expressed after 60 min (Fig. 5B),
368 suggesting an increase in H₂O₂ production and confirmed by its direct quantification (Fig. 6A).
369 Interestingly, this was concomitant with the increased expression of *TCH1* (Fig. 4A) that is a
370 marker of an increase in cytosolic calcium concentration.

371 The expression level of catalase (*CAT2*), an essential pathway of bulk, low affinity H₂O₂
372 detoxification (Gill and Tuteja, 2010), was not affected by HF-EMF exposure (Fig. 5C),
373 whereas that of *APX1*, which finely regulates the H₂O₂ level due to its high affinity, was
374 significantly increased after 60 min (Fig. 5D), indicating a possible involvement of the
375 AsA/glutathione detoxification pathway. The total quantity of glutathione did not change after
376 exposure (Fig. 6B), as well as the GSH/GSSH ratio. Ascorbic acid (Fig. 6C, left) level did not
377 change either, while the dehydroascorbic acid was the only actor of the AsA/glutathione
378 pathway displaying a significant variation 60 min after exposure (Fig. 6C, right). Taken
379 together, these results showed that non-thermic HF-EMF trigger a low but significant increase
380 in H₂O₂ production which reflects more of a signal transduction mechanism, acting in synergy
381 with possible calcium movements as reported by calmodulin increase, than a stress response.
382 This observation is consistent with the general scheme proposed by Kaur et al. (2021) in which
383 the initial interaction of electromagnetic field with plants triggers both calcium movements and
384 ROS metabolism as initial events of signal transduction cascade that later cause changes in
385 plant development. Modifications in ROS production and/or scavenging were in fact reported
386 among others in wheat (Chen et al., 2009), mung bean (Singh et al., 2012), onions (Chandel
387 et al., 2017), duckweed (Tkalec et al., 2007) and *Arabidopsis* (Senevirathna et al., 2020). It is
388 also striking that quite similar changes in ROS metabolism have been reported after exposure
389 of plants to a static magnetic field (SMF) or to an extremely low frequency electromagnetic
390 field (ELF-EMF), despite the fundamental differences in the nature of these fields. Indeed,
391 exposing plants to static magnetic field higher than the geomagnetic field generally caused an

392 increase in H₂O₂ production and ROS-scavenging enzymes activities and H₂O₂ synthesis-
393 related gene expression (see the reviews from Maffei et al., 2014, Saletnik et al., 2022 and
394 Hafeez et al. (2023) for details). In contrast, exposing plants to near null magnetic field (much
395 lower to that of the geomagnetic field) resulted in a drastic reduction of H₂O₂ in roots and shoots
396 (5-folds after 24h), likely related to the up-regulation of gene expression of ROS scavenging
397 enzymes (Parmagnani et al., 2022). ROS metabolism is therefore an essential component in
398 plants' perception of the presence/absence of static magnetic fields, as well as of their
399 exposure to very high frequency electromagnetic fields.

400 **Conclusion**

401 We demonstrated here that a very high frequency electromagnetic field, which we have verified
402 as having no heating effects in plants, was able to induce rapid changes in the expression of
403 several genes involved in signal transduction or ROS metabolism, as well as the production of
404 hydrogen peroxide. This ability appears to be unique to plants, as it is clear that non-thermal
405 exposure does not elicit a molecular response in humans (Roux et al., 2010; Habauzit et al,
406 2014). These results, in addition to many previous observations, raise the question of the
407 relevance of SAR in plants, insofar as this quantity (which otherwise adequately accounts for
408 biological effects in animals) is based on the thermal effects induced by exposure to
409 electromagnetic fields. This major difference could be the consequence of the fundamental
410 contrast in the logic of development (Vian et al., 2007), as plants create large surfaces, which
411 forces them to immobility but favors interaction with the environment (in this case interaction
412 with electromagnetic waves), whereas animals create volume and comparatively little surface
413 with the environment (which favors their mobility). The interaction mechanism of the
414 electromagnetic field, which clearly does not depend on thermal effects, remains as yet
415 misunderstood although several hypotheses have been proposed (Cifra et al, 2011;
416 Romanenko et al, 2017). Studies are still needed, and we believe that a more comprehensive
417 knowledge of the molecular and biochemical changes (such as those provided by an RNA
418 sequencing or metabolic analyses) taking place after non-thermal exposure could allow us to

419 make effective progress in understanding the mechanism of interaction between plants and
420 electromagnetic field.

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422

423 **Figure legends**

424

425 **Fig. 1:** experimental set-up. The electric polarization is indicated by a red arrow.

426 **A.** Schematic representation. The electromagnetic field was generated by a signal synthesizer,
427 amplified, and then emitted through a horn antenna placed at a distance $h=60$ cm above the
428 plants with an angle of 45° .

429 **B.** Picture of the set-up. Note that the experiments are performed in a shielded room, totally
430 isolated from the electromagnetic background noise.

431

432 **Fig. 2:** electromagnetic field characteristics

433 **A.** Field amplitude in the plant panel. The field amplitude was measured using a Narda EP-
434 601 triaxial probe in the center and at each corner of the plant panel.

435 **B. Frequency** Bandwidth of the exposure. A Fast Fourier Transform of the temporal signal
436 revealed that the bandwidth of about 30 MHz of the signal is centered on the fundamental
437 frequency (2.45 GHz) .

438

439 **Fig. 3:** Evolution of plant leaf temperature after EMF exposure.

440 **A.** The leaf surface temperature was continuously recorded from 2 min before the
441 application of the 100 kHz-modulated 2.45 GHz EMF field (a), during the 30 min
442 exposure between the two red vertical lines (b) and 3.5 min after the exposure stop (c).
443 The measurement was performed using a Lumasense Luxtron modèle STS
444 reflectometric probe. Representative recording out of 3 independent experiments.

445 **B.** Thermic imaging of the plant's panel before (left) of after (right) the application of a
446 30 min 100 kHz-modulated 2.45 GHz EMF. The red cross materializes the possibility

447 to measure selectively the temperature on a very reduced surface of the leaf.
448 Positioned on hot spots present on both unexposed and exposed samples, the
449 measures revealed similar surface temperatures (24.3°C and 23°C, on unexposed and
450 exposed samples, respectively).

451

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

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

460

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

462 Relative transcript accumulation of (**A**) NADPH oxidases *RBOHD* and (**B**) *RBOHF*,
463 (**C**) catalase *CAT2* and (**D**) ascorbate peroxidase *APX1* genes, all implicated in H₂O₂
464 production and scavenging, in unexposed (control) and exposed plants (15, 30 and 60 min
465 after 30 min exposure to HF-EMF at 2.45 GHz, SAR<0.21 WKg⁻¹). Data are means of n = 3
466 biological independent replicates ± s.e. Asterisks indicate significant differences after the non-
467 parametric Wilcoxon-Mann-Whitney test at the 95% confidence interval (*: P < 0.05; **: P <
468 0.01 and ***: P < 0.001) between the exposed and control condition for each different time
469 point.

470

471

472 **Fig. 6:** effect of HF-EMF exposure on ROS metabolism and MDA production

473 Amounts of **(A)** H₂O₂, **(B)** total glutathione (GSH, GSSG) and GSH/GSSG ratio, **(C)** amounts
474 of ascorbic (ASA) and dehydroascorbic (DHA) acids and **(D)** amounts of malondialdehyde
475 (MDA) in *Arabidopsis thaliana* leaves in unexposed (control) and exposed plants (60 min after
476 30 min exposure to HF-EMF at 2.45 GHz, SAR<0.21 WKg⁻¹). Data are means of n = 3
477 biological independent replicates ± s.e. Asterisks indicate significant differences after the non-
478 parametric Wilcoxon-Mann-Whitney test at the 95% confidence interval (*: P < 0.05; **: P <
479 0.01 and ***: P < 0.001) between the exposed and control condition.

480

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484 **Table S1: List of primer sequences used in this study.**

485 Genes in bold were used as housekeeping genes

Gene ontology	Gene name	Locus id	Primer sequences	Amplicon size	References
Glyceraldehyde-3-phosphate dehydrogenase	<i>GAPDH</i>	At1G13440	Forward: TCAGGAACCCTGAGGACATC Reverse: CGTTGACACCAACAACGAAC	174 bp	Jin <i>et al.</i> (2019)
Aldehyde dehydrogenase	<i>ALDH</i>	At3G66658	Forward: TGGGATATTTCCCTGCTCTG Reverse: GAAGAATCCGCAAGAACTGC	123 bp	Jin <i>et al.</i> (2019)
Calmodulin 1	<i>TCH1</i>	At5G37780	Forward: ATGGAAACGGCACTATCGAC Reverse: ATTGGTCATCACATGGCGTA	161 bp	newly designed
Zinc finger protein/stress responses	<i>ZAT12</i>	At5G59820	Forward: TGCGAGTCACAAGAAGCCTA Reverse: GTGTCCTCCCAAAGCTTGTC	127 bp	newly designed
Respiratory burst oxidase homolog D	<i>RBOHD</i>	At5G47910	Forward: CGTGGAGATCACGCTAGACA Reverse: AGGAGGTGGTGTGTTGAGG	149 bp	newly designed
Respiratory burst oxidase homolog F	<i>RBOHF</i>	At1G64060	Forward: GGTGCACCAGCACAAGATTA Reverse: TGAAATCCGAGATCGAATCC	148 bp	newly designed
Catalase 2	<i>CAT2</i>	At4G35090	Forward: CGAGGTATGACCAGGTTTCGT Reverse: CTCCAGGCTCCTTGAAGTTG	114 bp	newly designed
Ascorbate peroxidase 1	<i>APX1</i>	At1G07890	Forward: GCACTATTGGACGACCCTGT Reverse: AGCAAACCCAAGCTCAGAAA	117 bp	newly designed

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492

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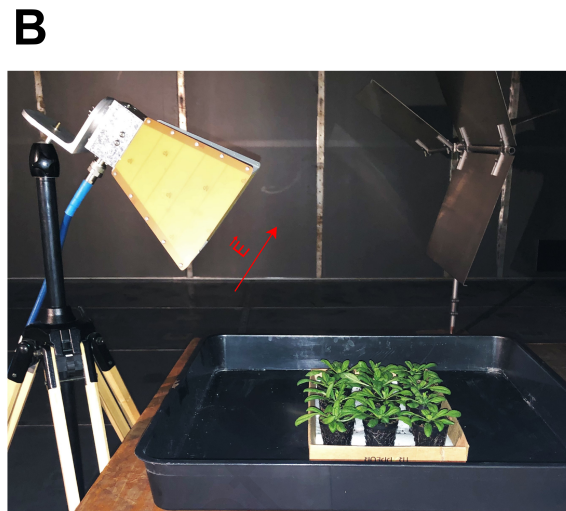
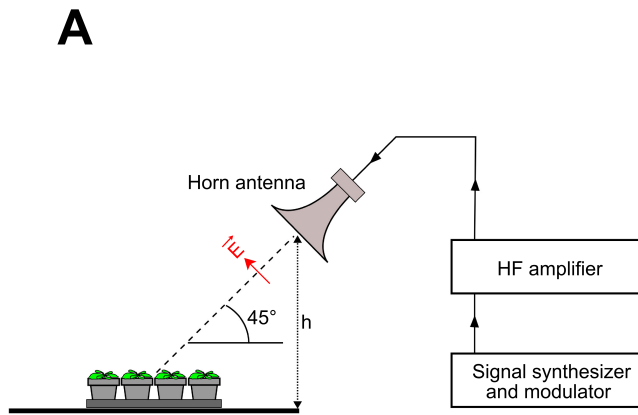
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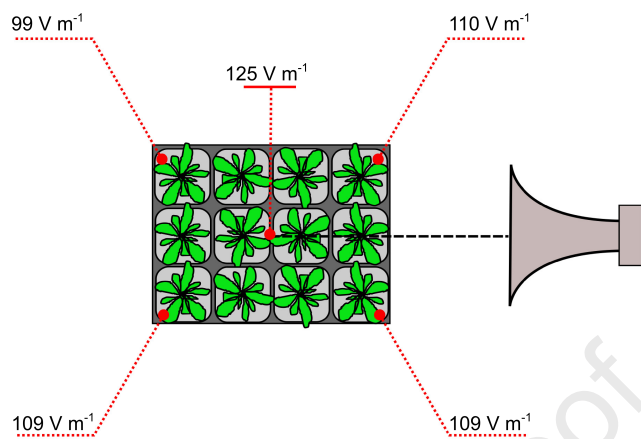
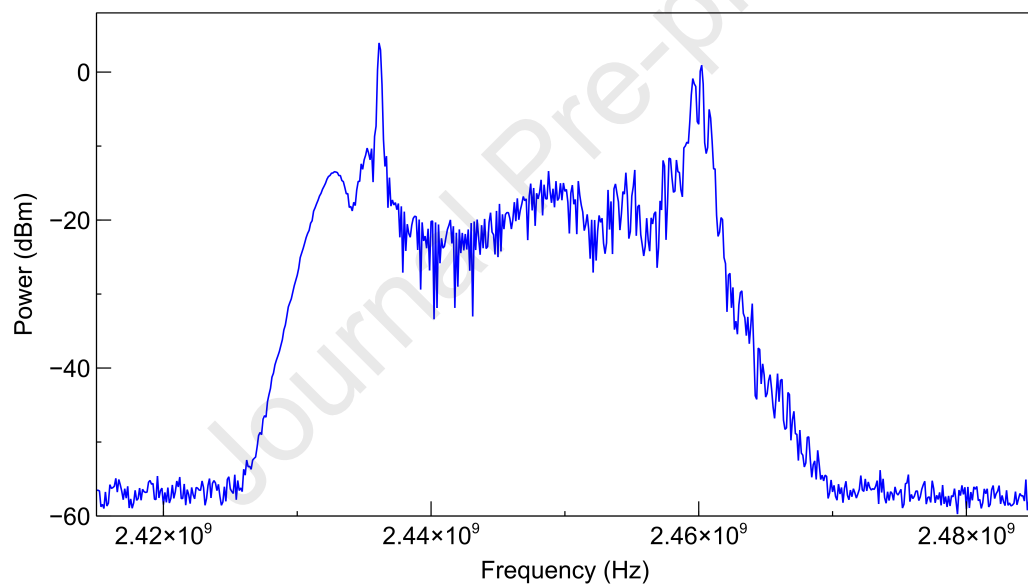
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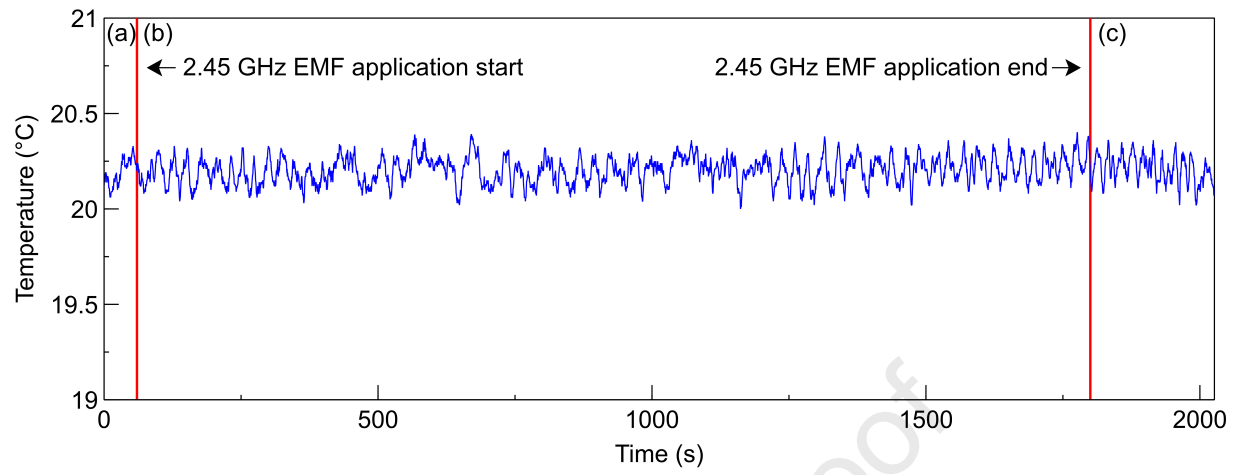
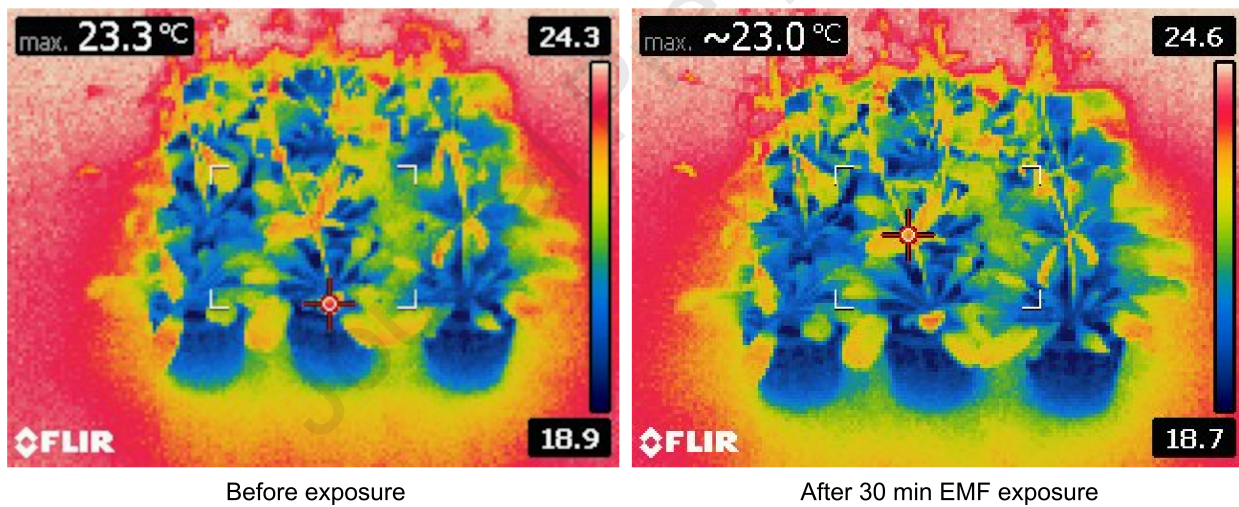
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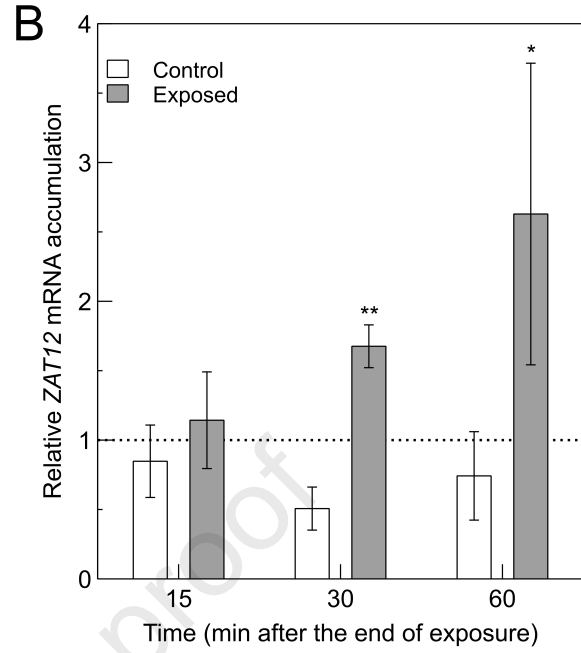
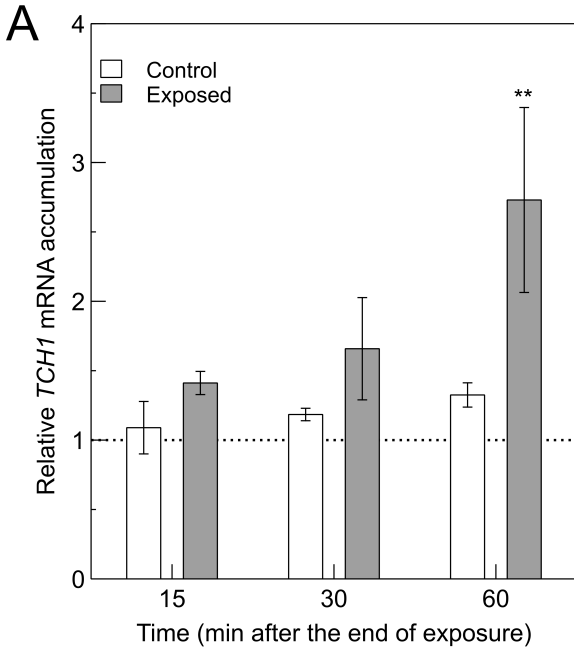
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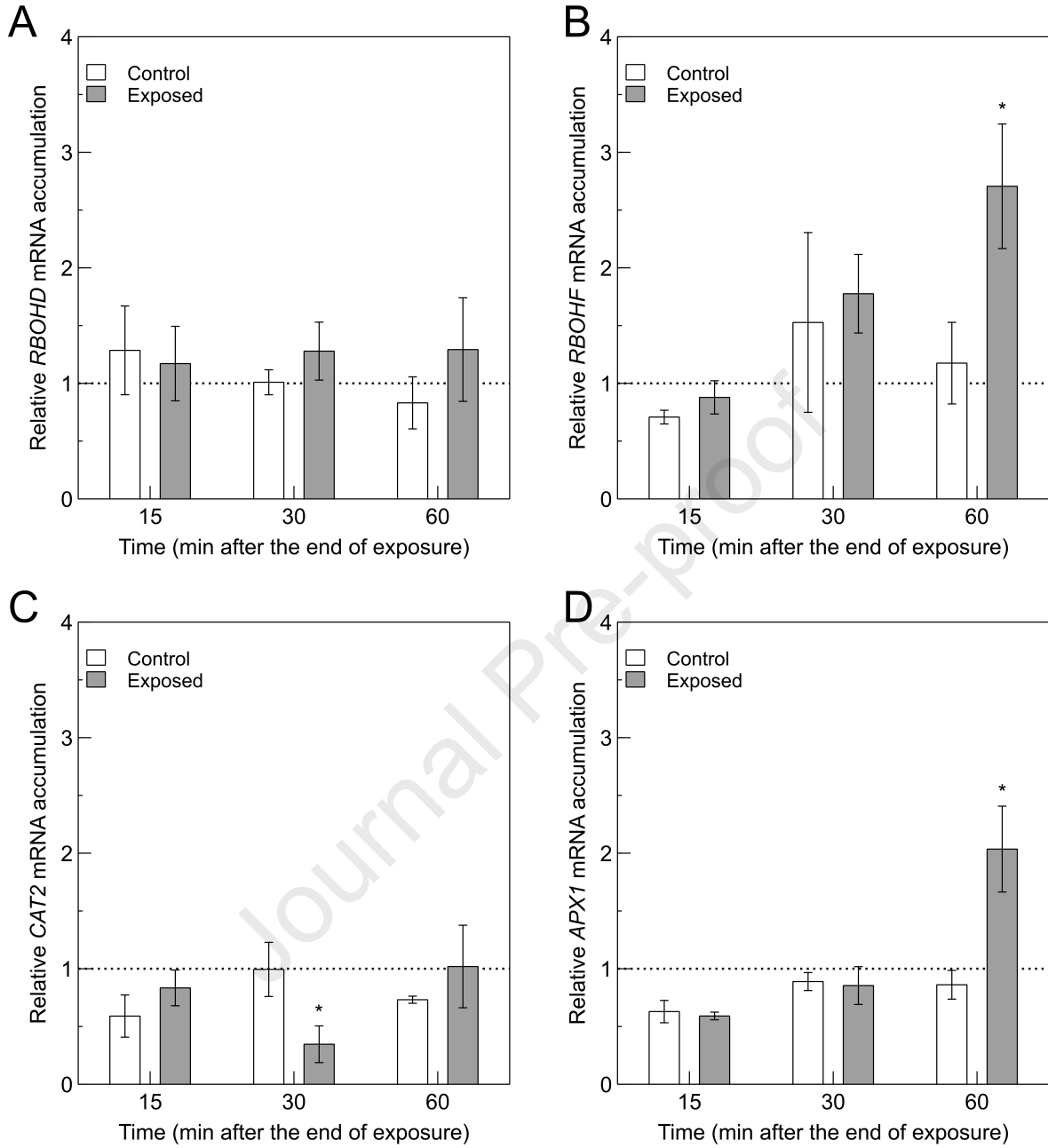
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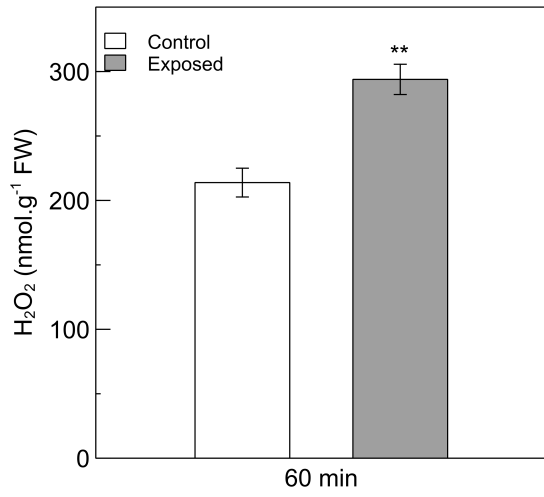
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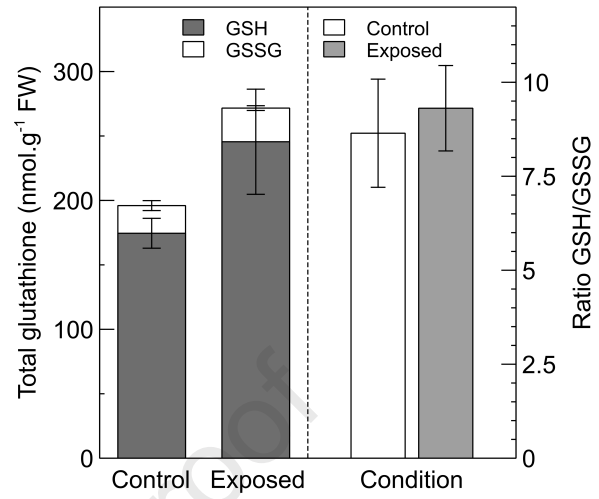




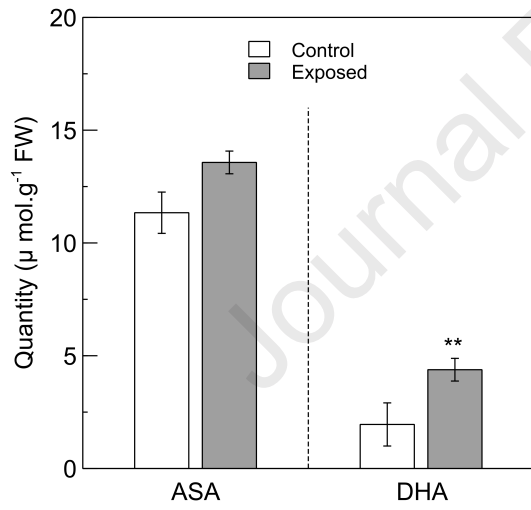
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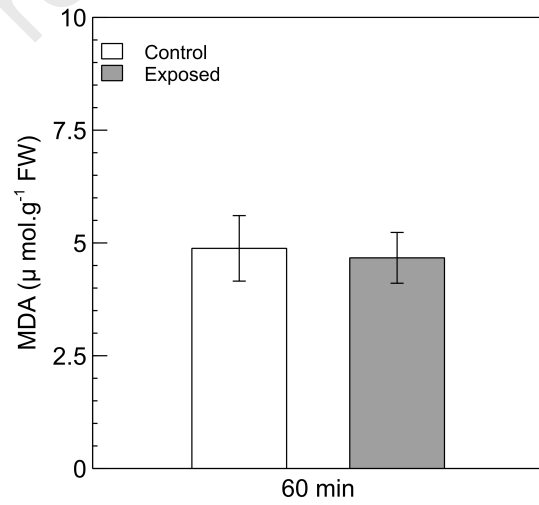
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Highlights

- In our condition, 2.45 GHz HF-EMF exposure did not induce thermal effect in plants
- We observed rapid and transient changes of gene expression and H₂O₂ metabolism
- Non-thermal biological responses to EMF exposure do exist in plants

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Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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