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Oxidative stress and an animal neurotransmitter synthesizing enzyme in the leaves of wild growing myrtle after exposure to GSM radiation

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ABSTRACT

Mobile phones turned to be the most common form of public communication. Therefore, life on our planet continues wrapped in a "cloud" of non-ionizing radiations. *Myrtus communis* L. is an evergreen shrub, common in Mediterranean formations, exposed and adapted to two seasonally separated and qualitatively different environmental stresses. Thus, we considered it important to investigate the response of this tolerant species to emitted GSM non-ionizing radiations and compare it to already available data from thoroughly investigated plant species. Although the leaves of the exposed plants present unaffected tissue arrangement, their mesophyll cells accumulate large amounts of secondary metabolites, their photosynthetic pigments are dramatically reduced, the ROS counted are significantly increased and the presence of DDC, which cannot be detected in the leaves of the control plants, is recorded in high levels. The exposed leaves seem to experience a severe oxidative stress which probably induces DDC expression and the biosynthesis of the neurotransmitter dopamine, the activation of the shikimate pathway and, eventually, the accumulation of secondary metabolites.

1. Introduction

Considering that Earth's natural radiofrequency environment has remained more or less unaltered within the lifespan of modern trees since before 1800, the major components of this environment were the radio emissions from space (galactic noise), the electric discharges within the atmosphere (atmospheric noise), and the small Radio Frequency (RF) component from the sun (Haggerty, 2010). Thus, we may assume that plants have evolved learning to use these environmental signals and intercept electromagnetic radiation (light) in order to regulate their circadian and seasonal functions (Haggerty, 2010). This is probably the main reason for which the impact of radiofrequency radiation on plants has received little attention and the physiological effects have been, rather arbitrarily, considered as negligible (Waldmann-Selsam et al., 2016).

High frequency electromagnetic fields (HF-EMF, i.e., frequencies from 300 MHz to 3 GHz, wavelengths from 1 m to 10 cm) are mainly human-produced, non-ionizing electromagnetic radiations that do not naturally occur in the environment (Vian et al., 2016). They are increasingly present because of the rapid development of wireless technology, such as Wi-Fi, various kinds of connected devices and cellular phones. Concerning the later, as the twentieth century passed away, the number of the mobile phone users increased from about 2.2 to 5.9 billion between 2005 and 2011 (Choi, 2012). Approximately four mobile phone service providers exist in each given geographical area (Hyland, 2005). Consequently, the number of base stations was also increased to support the tremendous growth of mobile phone users (World Health Organization, 2006) thus high frequency non-ionizing electromagnetic fields (HF-EMF) are increasingly present in the environment and the living organisms of the civilised world thrive within a "cloud" of non-ionizing radiations.

Much concern is given to the effects of this radiation to human life and environmental health (Roux et al., 2006; Pietruszewski et al., 2007; Sheridan et al., 2010; Sharma and Parihar, 2014) although radiofrequency constitutes a genuine environmental stimulus for plants as well. This stimulus is able to evoke specific responses many of them being similar to those observed after a stressful treatment (Waldmann-Selsam et al., 2016). Moreover, plants proved an outstanding model for studying such interactions since their architecture (high surface/volume ratio) optimizes their interaction with the environment. They remain exposed to radiation at a constant orientation in the electromagnetic field, due to their inability to move (Vian et al., 2016), while it is possible to easily document changes over time, such as disturbed cell structure, growth and yield (Stefi et al., 2017a, 2017b), dying

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branches and colour changes of leaves. These damages can be considered objective and cannot be attributed to psychological or psychosomatic factors (Waldmann-Selsam et al., 2016).

During the previous century and currently, several researchers have investigated the effects of electromagnetic radiation on plants, both in the laboratory (Harte, 1950, 1972; Jerman et al., 1998; Lerchl et al., 2000; Sandu et al., 2005; Roux et al., 2006; Pietruszewski et al., 2007; Tkalec et al., 2005, 2009; Beaubois et al., 2007; Haggerty, 2010; Pesnya and Romanovsky, 2013; Sharma and Parihar 2014; Cammaerts and Johansson, 2015; Kumar et al., 2015; Stefi et al., 2016, 2017a, 2017b), and on the field (Bernatzky, 1986; Volkrodt, 1987, 1991; Selga and Selga, 1996; Balodis et al., 1996; Haggerty, 2010). For both types of studies, most of the times, detrimental effects have been reported, even for weak radiation exposure (Halgamuge, 2017).

It has long ago been documented that mediterranean plants – both the evergreen sclerophylls (Mitrakos and Christodoulakis, 1981) and the seasonally dimorphics (Christodoulakis, 1989) – are exposed to two seasonally separated and qualitatively different environmental stresses (Mitrakos, 1980). In the summer, the exclusionary high temperatures and the arid environment, force plant metabolism to shift from the photosynthetic to other, secondary metabolic pathways to relieve the cell from the oxidative stress caused by the ionized P680 pigments. During winter, frost is the other stressing condition which seems to be treated in a different way by the evergreen sclerophylls compared to the seasonally dimorphics.

In spite of the rather hostile climate, the Mediterranean evergreen sclerophyllous plants are considered to be very productive (Mooney et al., 1970; Di Castri and Mooney, 1973; Mooney and Dunn, 1976). This is true only when the environmental conditions are mild. Yet, the Mediterranean climate is not so generous (Mitrakos, 1980; Mitrakos and Christodoulakis, 1981) and the periods of favorable conditions, when growth can be promoted, are limited and unpredictable (Margaris, 1981). Eventually, Mediterranean plant formations fall short in biomass production. This is mainly due to the stressing conditions of the long and arid Mediterranean summer. Among them is the excess of solar radiation during the summer months. Excessive solar radiation causes photodamage, disables photosynthesis and strongly reduces growth and productivity (Di Castri and Mooney, 1973; Margaris, 1981). This is the reason why an investigation on the effect of the recently, massively introduced, additional EM radiation, produced by the numerous cellphone towers settled throughout the urban areas and the countryside, covering 95% of the Greek territories, seems more than interesting. Moreover, the global debate on the effects of the EM radiation on animal and plant life is continuously boosted and new data are always welcomed.

Myrtle (family: Myrtaceae) is an evergreen sclerophyllous species well adapted to the Mediterranean climatic conditions. It is a shrub or small tree thriving in typical Mediterranean formations, common all over Greece's lowlands thus very often exposed to non-ionizing electromagnetic radiation produced by the numerous phone musts of the continuously expanding cellular communication system.

The production and accumulation of secondary metabolites, phenolics in particular, is a common feature of the mediterranean plants (Christodoulakis, 1992, Fahn and Cutler, 1992). Secondary metabolites do have a multi-protective role and stressing conditions enhance the biosynthesis of these metabolites which then appear abundant in the vacuoles all leaf cells of the plant (Mitrakos and Christodoulakis, 1981).

After exposure to the summer drought stress, myrtle follows the habit of almost all mediterranean species (Nikolakaki and Christodoulakis, 2007), concerning the activation of the shikimic acid pathway. This metabolic pathway eventually leads stressed plants to produce various secondary metabolites whose biosynthesis is shared by animal and plant organisms (Tocher and Tocher, 1972; Iriti, 2013) including animal neurotransmitters i.e. acetylcholine in *Urtica* spp (Jaffe, 1970; Barlow and Dixon, 1973; Odjakova and Hadjiivanova, 1997). In myrtle, these metabolites are accumulated either within the schizolysigenous gland cavities of the leaves or, mainly, in the form of phenolic tannin precursors or various types of tannins, within the vacuoles of all living cells, mostly those of the leaves.

Therefore, a) willing to exploit the numerous pieces of data on the stress response of the Mediterranean evergreen sclerophylls, b) having in mind that non-ionizing radiation proves to be a stressing factor for the plants by interfering with their secondary metabolic pathways, c) considering myrtle as a plant of wide distribution within the areas of Mediterranean climate, we set up a series of experiments to investigate the response of this plant to the radiation produced from a Global System for Mobile communications (GSM – mobile telephone) unit and work out on the results hoping to contribute to the reply on a global question mark.

2. Materials and methods

2.1. Plant material and radiation setup

Two myrtle individuals growing in a formation of Mediterranean evergreen sclerophylls, at the western slope of mount Hymettus (37°58′00.80′′N, 23°47′07.35′′E, altitude 259 m), north-East of Athens metropolitan area, were selected and young leaves of the previous vegetative period (autumn 2016) were marked with plastic tags each year.

Soon as the leaves reached their maturity (20 days after their emergence), the radiation procedure was applied. Leaves were irradiated with a GSM-1800 MHz mobile phone for 30 min, in talk mode, every Monday, Wednesday and Friday, at 08:00 am, from **02 Nov, 2016** until **21 Dec 2016** (1st experiment – 49 days) and **20 Feb 2017** until **10 Apr 2017** (2nd experiment – 50 days). The hand set (Motorola C115) was mounted on a gooseneck holder with a clamp at the end of the flexible extension arm which facilitated positioning the apparatus in touch with the exposed leaves. A tiny, remote controlled, battery operated transistor radio (Panasonic RF-H830) was also mounted facing the phone in order to provide continuous sound, simulate the talking mode and activate the handset to transmit towards the regional phone mast. The radio was tuned to a distant radio station; thus, the sound produced was actually full of radio parasites, a practically constant noise at high volume. The whole construction was tightly fixed on a heavy duty photographic tripod (Fig. 2 in Plate 2). The average E-field intensity value was **27 V/m**, for 6 min (according to ICNIRP, 1998). Maximum value of 58 V/m was recorded while background records were as low as 0,650 V/m. Measurements were taken with a NARDA SRM3000 (Germany) spectrum analyser while the GSM unit was transmitting. Supplementary measurements, for cross-checking, were taken with a broadband field meter (TES-92, 50 MHz - 3.5 GHz, Electromagnetic radiation detector – TES Electrical Electronic Corp. Taipei, Taiwan, R.O.C.). Values recorded were identical.

Investigation of the leaves was scheduled and executed as indicated in Fig. 1.

2.2. Leaf anatomy – microscopy

At the end of each experiment and when the plants had been exposed to twenty-two (22) doses of radiation, five leaves from the control and five from the exposed plants were cut-off, in random, a piece from the centre of each leaf was detached (the central nerve removed), cut into numerous small pieces (1 × 1 mm) and fixed in phosphate buffered 3% glutaraldehyde (pH 6.8) at 0 °C for 2 h. The tissue was post fixed, overnight, in 1% osmium tetroxide in phosphate buffer. A few pieces from the fixed tissue were dehydrated in graded acetone series, critical point dried, coated with gold and viewed with a JEOL JSM-6530 Scanning Electron Microscope. The rest of the tissue was dehydrated in graded ethanol series and embedded in Durcupan ACM (Fluka, Steinheim, Switzerland). Semithin sections, obtained from a LKB Ultrotome III, were placed on glass slides and stained with 0.5 toluidine blue O (in 1% borax solution), as a general stain, for light microscopic

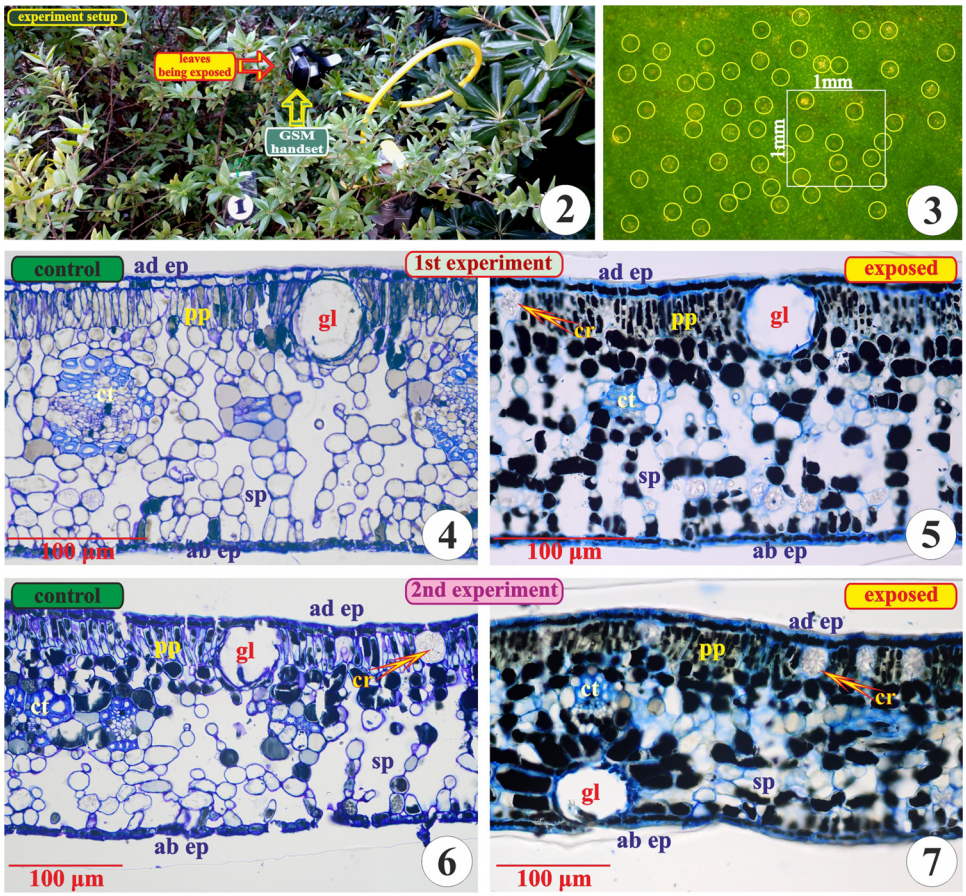

Plate 2. (Figs. 2–7): Experiment set-up, gland count on fresh whole mounts and cross sections from the two leaf types (both experiments) of myrtle. **Fig. 2.** The GSM handset, the marked myrtle individual (1, at the bottom) and the leaves under exposure are demonstrated; **Fig. 3.** Low magnification micrograph of a leaf whole mount. Glands are marked with circles; **Fig. 4.** Cross section of a control leaf (1st experiment). A gland can be observed just below the adaxial epidermis; **Fig. 5.** Cross section of an exposed leaf (1st experiment). The mesophyll cells, impregnated with osmiophillic material and a gland, just below the adaxial epidermis, can be observed; **Fig. 6.** Cross section of a control leaf (2nd experiment). A gland and a crystal can be observed just below the adaxial epidermis; **Fig. 7.** Cross section of an exposed leaf (2nd experiment). The mesophyll cells, impregnated with osmiophillic material and a gland, just below the abaxial epidermis and a few crystals below the adaxial epidermis, can be observed. **ad ep**: adaxial epidermis; **ab ep**: abaxial epidermis; **pp**: palisade parenchyma; **sp**: spongy parenchyma; **ct**: conductive tissue; **gl**: secretive structure; **cr**: crystal.

observations. Ultrathin sections were placed on 100 mesh grids, double stained with uranyl acetate and lead citrate (Reynolds, 1963) and viewed with a Phillips EM-300 Transmission Electron Microscope.

The whole procedure was repeated at the end of each experiment and the leaf tissue was subjected to identical treatment, i.e. fixed, dehydrated, embedded, sectioned, stained and observed. The results were thoroughly recorded for an oncoming evaluation and comparison. Literature for double fixation is cited in detail by Christodoulakis et al., (2009) and Christodoulakis et al., (2010).

The procedures for protein extraction and determination of protein concentration where repeated: 1) the 1st day, after the first radiation dose, 2) at the middle of the exposure period when the leaves had been exposed to 11 doses of radiation and 3) at the end of the experiment, when the radiation process was fulfilled (22 doses) (Fig. 1).

2.3. Histochemistry

Fresh leaves were sectioned by hand or with a cryotome (Leica CM1850, Germany) at −10 °C and stained with histo-chemical reagents: (a) osmium tetroxide for unsaturated lipids; (b) antimony trichloride ($SbCl_3$) for terpene containing steroids; (c) vanillin/HCl for flavonoids; (d) concentrated H_2SO_4 for sesquiterpenes; (e) Wagner's reagent for alkaloids; (f) Dittmar's reagent for alkaloids; (g) iodine–iodide solution for alkaloids; (h) potassium bichromate for tannins; (i)

1 dose/1st day			11 doses/24 days			22 doses/49 days		
preliminary 1st experiment (2 Nov 2016 to 21 Dec 2016) – **anatomical investigation**								
			25 November 2016 **leaf morphology**			21 December 2016 **leaf morphology, tissue fixation & microscopy (OM, SEM, TEM)**		
2nd experiment (20 Feb 2017 to 10 Apr 2017) – **anatomical and biochemical investigation**								
20 February 2017			15 March 2017 **leaf morphology**			21 April 2017 **leaf morphology, tissue fixation & microscopy (OM, SEM, TEM)**		
immediately	1 hr after exposure	24 hrs after exposure	immediately	1 hr after exposure	24 hrs after exposure	immediately	1 hr after exposure	24 hrs after exposure
ROS pigments DDC			**ROS pigments DDC**			**ROS pigments DDC**		1

Fig. 1. Timetable for the investigation of the myrtle leaves (both experiments).

DMB (dimethoxy benzaldehyde or veratral aldehyde) for phenolic tannin precursors; (j) ferric chloride for polyphenols; (k) alcoholic vanillin/HCl (vanillin test) for phenolic compounds; (l) 4-nitrosophenol in concentrated H_2SO_4 for monoterpene phenols; (m) nitroso reaction for catechol tannins. All stains were matched by controls. The literature for histochemical reactions is cited in detail by Christodoulakis et al. (2009) and Christodoulakis et al. (2010).

2.4. Protein extraction and determination of protein concentration

Leaves from both control and exposed samples were detached and grinded in liquid nitrogen. Protein extraction was performed with RIPA lysis buffer (Thermo Fisher Scientific Inc. – NYSE: TMO), samples were centrifuged (12.000 × *g*, 30 min, 4 °C) and the supernatants were stored at −20 °C. Total protein concentration was determined according to the method of Bradford (Bradford, 1976), using Bovine Serum Albumin (BSA) as standard.

2.5. SDS-polyacrylamide gel electrophoresis and immunoblotting

SDS-polyacrylamide gel electrophoresis and immunoblotting SDS-PAGE was performed on a "Biorad Mini Protean" electrophoresis apparatus as described by Laemmli (1970), using a 12% polyacrylamide slab gel. Electrophoresis was carried out at 120 V for 2 h at room temperature. Following electrophoresis, samples were transferred onto nitrocellulose membrane (Towbin et al., 1979). Immunological detection of the proteins was performed according to Batteiger's method (Batteiger et al., 1982) and subsequent immunological detection was performed using the antibodies described below.

2.6. Antibodies

A specific polyclonal L-**D**opa **DeC**arboxylase (DDC) antibody raised against the purified human protein (Mappouras et al., 1990) and the DDC antibody raised from *Ceratitis capitata* (lab. of Prof. Dr D. Vassilacopoulou, University of Athens, GR – Bossinakou and Fragoulis, 1996) were used for immunodetection.

2.7. Pigments protocol

Chlorophyll pigments, from approximately 50 mg leaves, were extracted with 1 ml 80% acetone, overnight, at 4 °C. Supernatant was transferred to a 1-ml glass cuvette for measurement in a Shimadzu Europe UV-1700 spectrophotometer. Absorbance was recorded throughout the visible spectrum, from 400 to 700 nm. We focused at both 663.6 and 646.6 nm, corresponding to *chlorophyll –a* and *chlorophyll –b* respectively and at 625 nm for the absorbance of *chlorophyll –c*. Quantification of pigment content was calculated using molar extinction coefficients specifically for this method (Gechev, 2013). Chlorophyll –a, $e_{663.6} = 76.79$ and $e_{646.6} = 18.58$; chlorophyll –b, $e_{663.6} = 9.79$ and $e_{646.6} = 47.04$. The total chlorophyll content is calculated using the following formula and normalize per fresh weight: Chlorophyll a + b = $19{,}54A_{646.6} + 8{,}29A_{663}$. For beta carotenes and xanthophylls no formula is available for quantification; only the absorbance value is utilized.

2.8. Total ROS estimation

ROS levels were measured using 10 mM of the oxidant sensitive fluorescent acetyl ester CM-H_2DCFDA (5-(and-6)-chloromethyl-2′, 7′-dichlorodihydrofluorescein diacetate) dissolved in DMSO. CM-H_2DCFDA is a general oxidative stress indicator that can enter cells by penetrating the cell membrane through passive diffusion. Inside the cell its acetate groups are cleaved by intracellular esterases and oxidation by ROS leads to the formation of fluorescent DCF product, which can be detected via fluorometry. The procedure was the following: Tissues were homogenized in liquid nitrogen, placed in a 2 mL tube with 1 mL of 10 mM Tris–HCl, pH 7.2 and centrifuged at 12,000 × *g* for 20 min at 4 °C. Supernatant transferred to a fresh 2 mL tube and subsequently, the samples were placed continuously in incubator for 30 min with CM-H_2DCFDA diluted in 10 mM Tris–HCl at 37 °C in the incubator. Then, the ester was removed and further incubation for 20 min in a Tris–HCl followed. Finally, tissues were washed three times with PBS buffer solution.

Measurement of fluorescence was made at the supernatant with VersaFluor Fluorometer System (Bio-Rad, 170- 2402, Hercules, CA) with excitation filter at 490 nm and emission at 520 nm. For the recovery experiments, various time points were tested as trials before finalizing the most promising values. In every set of experiments, duplicated samples were used for the exposed samples and the same run included control samples. Total ROS was expressed as fluorescent units/μg of protein extracts (Sunkar, 2010).

3. Results

3.1. Morphology and microscopy

Control and exposed leaves were carefully examined for their external morphology. Exposed leaves, during the radiation process and at the end of the experiment, did not seem to be affected since their magnitude, shape, epidermal tissue arrangement and colour, appeared identical to their control counterparts.

Schizolysigenous gland distribution, microscopically measured on fresh leaves (Fig. 3 in Plate 2) (glands/mm^2: 8,2 ± 0,8 for the control and 9,1 ± 0,6 for the exposed leaves of the 1st experiment; 9,1 ± 0,7 for the control and 9,4 ± 0,7 for the exposed leaves of the 2nd experiment) as well as stomatal frequency (stomata/mm^2: 451 ± 28 for the control and 459 ± 35 for the exposed leaves of the 1st experiment; 438 ± 31 for the control and 445 ± 39 for the exposed leaves of the 2nd experiment), measured at low magnification OM (Fig. 3 in Plate 2) and SE micrographs, do not seem to vary significantly in the two leaf types.

Cross sections of both leaf types were investigated in detail (Fig. 4–7 in Plate 2). An obvious difference has to do with the leaf compactness and the accumulation of secondary metabolites in the cells of the mesophyll tissues (compare Figs. 4–7 in Plate 2). Exposed leaves appear more compact while palisade and spongy parenchyma cells accumulate large amounts of osmiophilic material (Figs. 5 and 7 in Plate 2).

Cross sections of fresh leaves histochemically treated with DMB (dimethoxy–benzaldehyde), a reagent used to localise phenolic tannin precursors, reacted positively giving a vivid red colour for the cells of both adaxial and abaxial epidermis (Fig. 8 in Plate 3) as well as for the secretory cells of the glands (Figs. 8 and 9 in Plate 3). An intense brown tincture was obtained, in the mesophyll cells, after using potassium bichromate for tannins. Ferric chloride for polyphenols stained the epidermal and most of the mesophyll cells with a dark blue colour, nitroso – reaction for catechol tannins stained the mesophyll brown while a deep purple colour was obtained with alcoholic vanillin/HCl, a stain for phenolic compounds in general.

Scanning electron microscope observations confirmed the findings from the light micrographs since the mesophyll cells of the exposed leaves appear impregnated with a dense material (compare Figs. 14 and 15 in Plate 4).

A clear image was obtained from the Transmission Electron Microscopic observations. The mesophyll cells of the exposed leaves are tightly "packed" with secondary metabolites (sm in Figs. 16 and 17 in Plate 4) while the mature chloroplasts of these cells demonstrate their productive past by exhibiting the large starch granules they have accumulated (st in Figs. 16 and 17 in Plate 4). All membranes and organelles of the cells from the exposed leaves (i.e. mitochondria) seem well preserved without any distinguishable deformations.

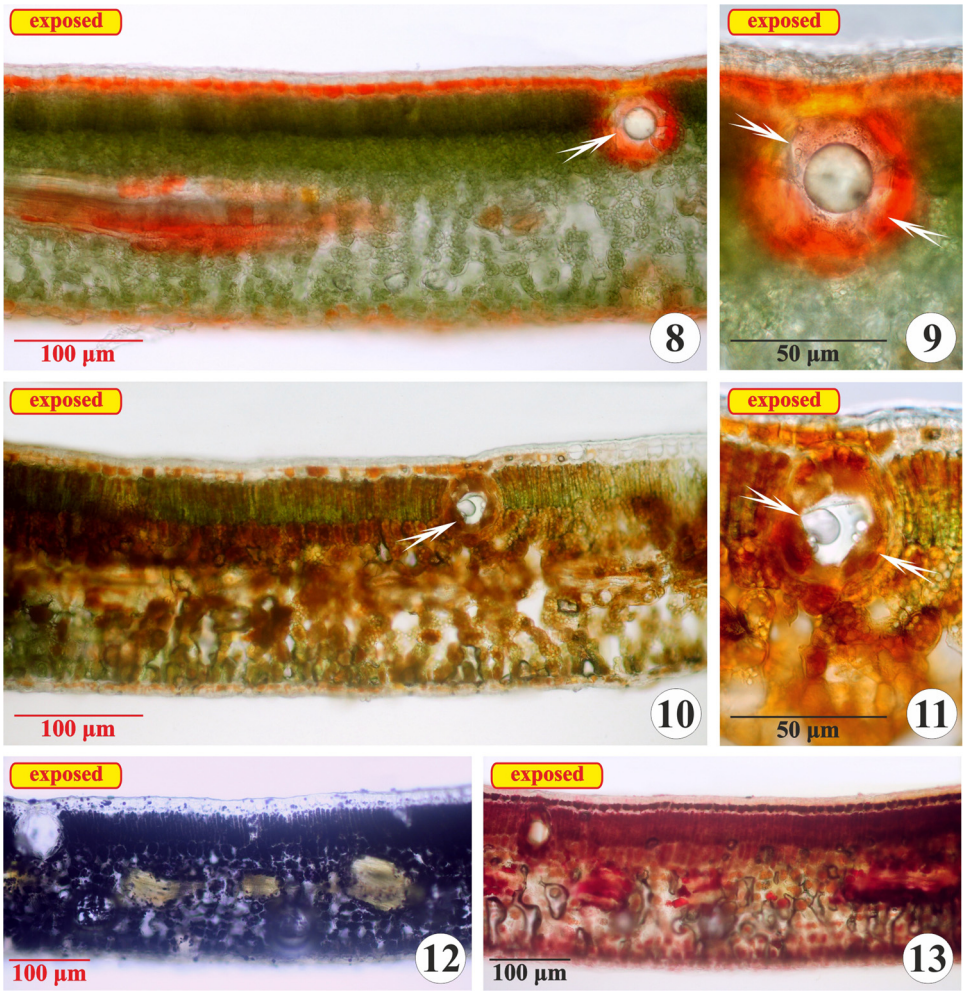

Plate 3. (Figs. 8–13): Cross sections of fresh myrtle leaves treated with histochemical reagents. **Fig. 8** and **Fig. 9**, treated with DMB. **Fig. 10** and **Fig. 11**, treated with $K_2Cr_2O_7$. **Fig. 12**, treated with $FeCl_3$ solution. **Fig. 13**, treated with alcoholic vanillin/HCl. White arrows point at the secretory, epithelial cells of the glands.

3.2. Pigments and ROS estimation

For the photosynthetic pigments the measurements were executed three times: once after the first exposure to radiation, then in the middle (24 days) of the radiation treatment and, finally, after the end of the exposure series (49 days). Each time the pigments were measured immediately after irradiation, one hour after irradiation and 24 h after irradiation. The absorption graphs are presented in Fig. 18, 20 and 22 in Plate 5 (left column).

It seems that, as time goes by, the absorbance graph dives to lower values. The minimum absorbance is observed 24 h after irradiation. Surprisingly a new peak appears, in every graph, at about 535 nm (Fig. 18, 20 and 22 in Plate 5), an area in the band where none of the photosynthetic pigments absorbs. This confined region of the band is "reserved" for the absorbance of the anthocyanins (537–542 nm – Merzlyak et al., 2008). Photosynthetic pigments do not seem to be affected during or immediately after irradiation. Yet they are reduced one hour after radiation and present a significant downfall, 24 h after their exposure (Fig. 18, 20 and 22 in Plate 5).

Concerning measurements of Reactive Oxygen Species (ROS) (Figs. 19, 21 and 23 in Plate 5) it seems that immediately after irradiation the difference between control and exposed leaves remains within the limits of the statistical error. Yet, as time lapses, the oxidative stress seems to increase. One hour after irradiation, ROS counts are almost 30% more in the exposed leaves, in all three cases, while after 24 h the increase of ROS climbs to about 50% compared to the control leaves (Figs. 19, 21 and 23 in Plate 5).

3.3. Secondary metabolites of special interest

Our investigations with SDS-PAGE electrophoresis are presented in Figs. 24–26 in Plate 6. Samples from the control leaves were compared to samples from their exposed counterparts. Exposed leaves treated for immunoblotting immediately after irradiation, do not present any detectable alteration compared to the control ones, concerning the expression of DDC. On the other hand, exposed leaves, one hour after irradiation, seem to express DDC at unexpectedly high levels. This can clearly be demonstrated in Figs. 24–26 in Plate 6 where the expression of DDC appears cross checked with both antibodies (left column for anti-human DDC Ab and right column for anti-*Ceratitis capitata* DDC Ab). In Fig. 24 in Plate 6 DDC immunodetection appears at approximately 55 kDa at least one hour after irradiation (lane L3 in both columns). The expression of DDC remains at high levels in the exposed leaves even 24 h after irradiation (Fig. 24 in Plate 6, lane L4 in both columns). After 11 exposures (22 days) (Fig. 25 in Plate 6) and 22 exposures (49 days) (Fig. 26 in Plate 6) the same results appear for the expression of DDC one hour and 24 h after irradiation, for both antibodies used.

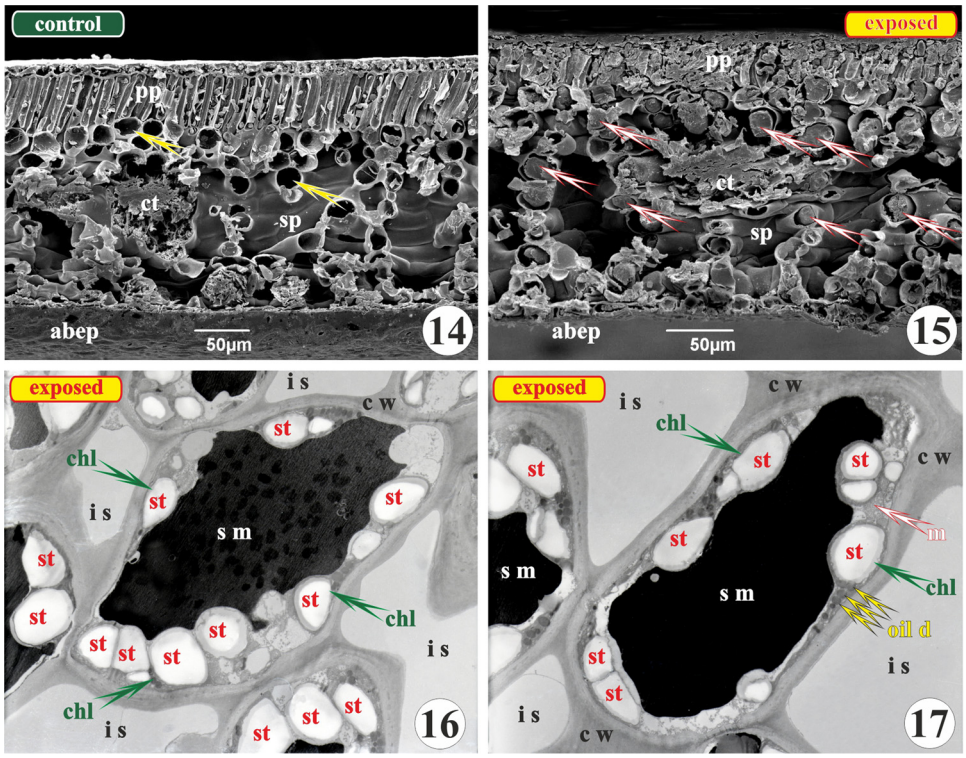

Plate 4. (Figs. 14–17): Scanning and transmission electron micrographs from myrtle leaves. Scanning EM: **Fig. 14.** The leaf of a control plant. Mesophyll cells appear free from inclusions (yellow arrows). **Fig. 15.** The leaf of an exposed plant, 24 hours after the last irradiation (22 exposures). Most of the mesophyll cells appear filled with dense material (white arrows). Transmission EMs from the mesophyll of **exposed leaves**: **Fig. 16.** Palisade cells obliquely sectioned. The central vacuole appears full of secondary metabolites (**sm**). Chloroplasts (**chl**) exhibit their productive past through the numerous starch grains (**st**). **Fig. 17.** Palisade cell with the central vacuole loaded with osmiophillic secondary metabolites (**sm**). Chloroplasts with starch and oil droplets and a few mitochondria can be observed. **abep**: abaxial epidermis; **pp**: palisade parenchyma; **sp**: spongy parenchyma; **ct**: conductive tissue; **st**: starch; **sm**: secondary metabolites; **cw**: cell wall; **oil d**: oil droplet; **chl**: chloroplast; **m**: mitochondrion; **is**: intercellular space.

4. Discussion

4.1. Microscopy

Irradiated mature leaves of myrtle appear unaffected after the experiment. Stomatal frequency remains the same in both leaf types. The frequency of the oil glands seems not to be affected as well. Yet, the production and accumulation of phenolic compounds within the vacuoles of the cells of the exposed leaves indicates the presence of an environmental stress.

Considering the results of the histochemical reactions as well as their osmiophilic nature, the texture and the dark colour of the vacuole inclusions as seen in the transmission electron micrographs, we may assume that the compounds accumulated are of phenolic nature. They must be either phenolic tannin precursors or condensed phenolics, at various degrees of condensation (i.e. hydrolysable tannins or non-recyclable tannins). The accumulation of phenolics, even as dead-end products, has long ago been considered as a very common feature of the mediterranean plants (Dement and Mooney, 1974; Orshan, 1989; Dafni, 1991; Fahn and Cutler, 1992; Christodoulakis, 1992; Meletiou-Christou et al., 1994; Stephanou and Manetas, 1997) – and not only (Ramakrishna and Ravishankar, 2011) – when sustaining environmental stress (drought or high temperature, high light intensity).

During our investigation, the environmental conditions were chosen, on purpose, to be moderate. No drought, no high temperatures, no high light intensity (winter months). In spite of these moderate conditions the accumulation of phenolics in the cells of the exposed leaves was remarkable compared to their control counterparts. Since the only stressing factor was radiation, we may assume that plants actually suffer when exposed to GSM radiation.

4.2. ROS

Increased activities of H_2O_2 and ROS in cells provide clear evidence for the vulnerability of plants to oxidative stress upon EMR exposure (Jayasanka and Asaeda, 2014). Reactive oxygen species are highly active compounds and excess availability in cells may lead to oxidation of membranes and various cellular components that ultimately result in the oxidative destruction of the cells (Mittler 2002; Hung et al., 2005). Oxidative stress is probably responsible for the production of phenolics which play an important role for plants under stress conditions (Weidner et al., 2009).

4.3. Absorbance

The measurement of photosynthetic pigments can provide basic information on the physiological status of a plant. High-pressure liquid chromatography (HPLC) is the "state of the art" method for this purpose since it provides an accurate determination of a variety of photosynthetic pigments simultaneously. Yet this technique has a drawback compared to conventional spectroscopic techniques since it is more prone to structural modification of pigments during extraction, thus generating erroneous results (Hu et al., 2013). In the current investigation, the extraction method used has already been cross checked in previous investigations (Stefi et al., 2016, 2017a, 2017b) for not producing any artifactual conversion of chlorophylls.

An interesting detail of this investigation is that besides the absorption of the photosynthetic pigments, which falls significantly 24 h after the last dose, a new peak appears: that of the anthocyanins. Anthocyanin absorption maxima in vivo, as estimated with spectrophotometry of intact anthocyanic leaves and micro-spectrophotometry of vacuoles in the leaf cross-sections, were found between 537 nm and 542 nm, showing a red shift of 5–20 nm compared to the corresponding maxima in acidic water–methanol extracts (Merzlyak et al., 2008). Anthocyanins are synthesized in order to absorb the excessive solar energy and prevent photo damage. They appear in stressed leaves (Merzlyak et al., 2008). In our investigation, they are present even in the control leaves probably because of the "Mediterranean habits" of these leaves which start their life "red coloured" and obtain their normal green colour at maturity (Manetas et al., 2002; Liakopoulos et al., 2006). Although it is not known whether anthocyanins can scavenge reactive oxygen in living plant cells (Gould et al., 2002) some data support the hypothesis that "red cells" have elevated antioxidant capabilities *in vivo* (Gould et al., 2002).

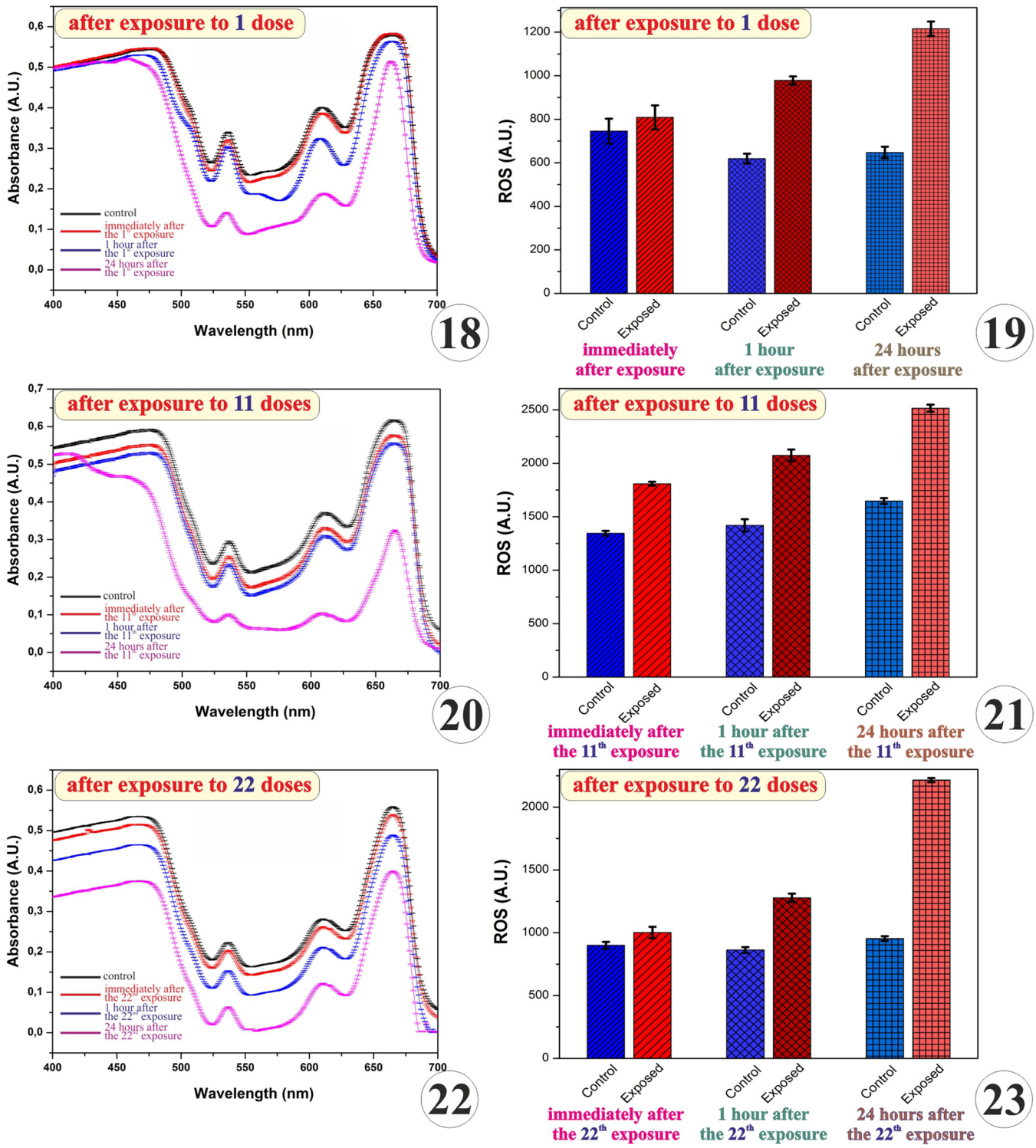

Plate 5. (Figs. 19–23): Values for the concentration of the photosynthetic pigments (left column, **Figs. 18**, **20** and **22**) and Reactive Oxygen Species – ROS (right column, **Figs. 19**, **21** and **23**) from the myrtle leaves investigated after exposure to 1 dose (first row, **18** and **19**), 11 doses (second row **20** and **21**) and 22 doses (third row, **22** and **23**).

Therefore, we may assume that since the stressing conditions of the Mediterranean climate were not present in our experiment, the drop of the photosynthetic pigment absorbance is due to radiation while the presence of anthocyanins is an indication that some other, non-detectable stressing factors have an impact on the leaves.

4.4. Secondary metabolite biosynthesis

L-DOPA is considered among the secondary metabolites whose biosynthesis is shared by animal and plant organisms (Tocher and Tocher, 1972; Iriti, 2013). These metabolites are involved, in concert with some phytohormones, in some regulatory functions in plant physiology such as growth, carbohydrate metabolism and some stress responses (Kulma and Szopa, 2007). The rapid, impressive increase of

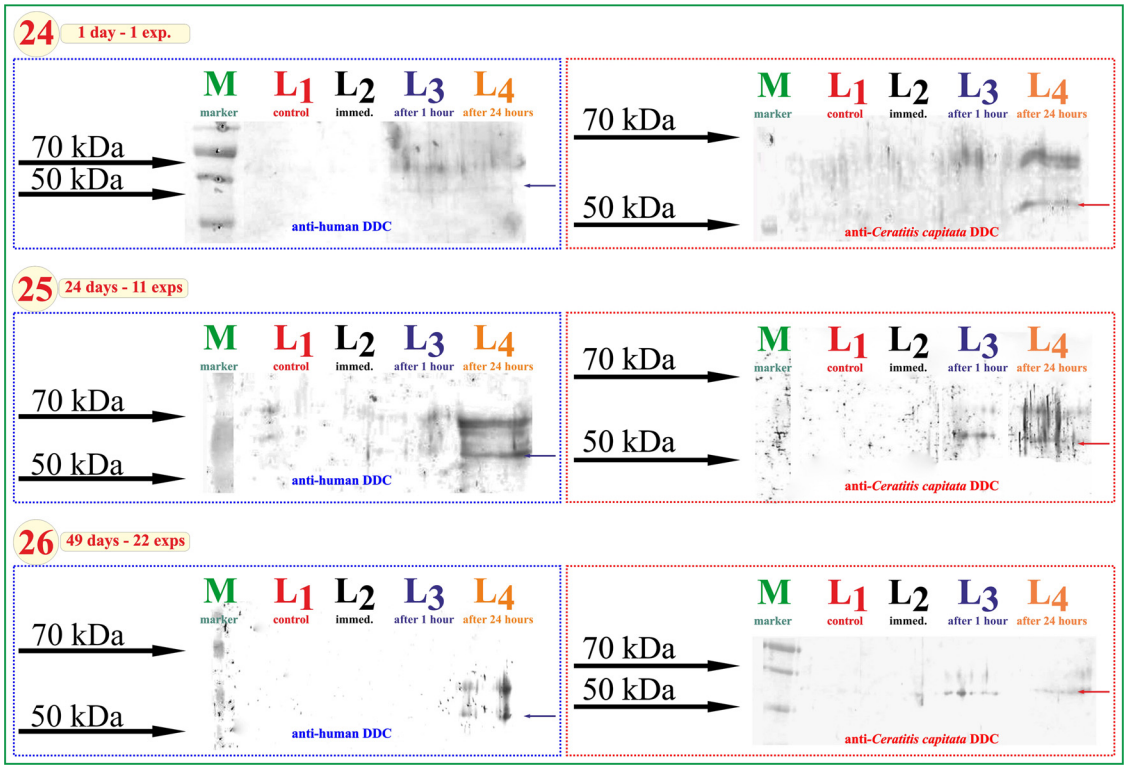

Plate 6. (Figs. 24–26): Expression of DDC in control and irradiated myrtle leaves. Left column: Immunodetection with anti-human DDC antibody. Right column: Immunodetection with anti-*Ceratitis capitata* DDC antibody. Immunodetection after, **Fig. 24.** the first exposure (immediately, one hour, 24 hours); **Fig. 25.** eleven exposures (immediately, one hour, 24 hours); **Fig. 26.** twenty-two exposures (immediately, one hour, 24 hours).

DDC expression in the leaves of the exposed plants indicates the urgent need for the elimination of the volume of its substrate, that is L-DOPA, through decarboxylation of L-DOPA to another well-known mammal neurotransmitter (Kulma and Szopa, 2007), the catecholamine *dopamine*, an 3,4-dihydroxy-subsituted phenyl ring possessing amine (Odjakova and Hadjiivanova, 1997). Dopamine is a secondary metabolite which can regulate, as most catecholamines, carbohydrate metabolism, serve as a precursor of some alkaloids, protect the plants against certain pathogens and is involved in nitrogen detoxification (Kulma and Szopa, 2007). Thus, the observed fluctuation of the DDC expression patterns could reflect a possible gene expression regulation event which governs the phenomenon revealed through our western blot experiments (Figs. 24–26 in Plate 6).

It can also help the plants defend themselves against detrimental climatic conditions, environmental pollutants, high solar irradiance and water deficit (Iriti, 2013). Considering all the above we may presume that the exposed leaves produce DDC and, consequently dopamine, because they experience an inevitable, severe environmental stress due to their exposure to radiation.

What seems very interesting in the whole experiment is that photosynthetic pigments, ROS and DDC expression appear not to be affected immediately but one hour after exposure and the detected expression continues to reflect the stress situation even 24 h after irradiation. Yet, it seems that before the next exposure, a recovery mechanism "resets" the cellular behaviour so that photosynthetic pigments return to their normal concentration, ROS drops down to normal and DDC expression is suppressed. This appears to be a marvellous mechanism of recovery which gives the plant the ability to sustain long term stress conditions although avoidance of the detrimental effects cannot be unlimited. The results of the current investigation are supported by a similar approach on the significance of the DDC gene family in poppy (*Papaver somniferum*) where the authors suggest that valuable clues may be provided by identifying the temporal, spatial, developmental and environmental cues that control expression of the various gene family members (Faccini and De Luca et al., 1994).

Our results are in total agreement with the various responses that have been observed after exposure to EMF at the cellular, molecular and whole plant scale. It is beyond any doubt that numerous metabolic activities (ROS metabolism, Krebs cycle, pentose phosphate pathway, chlorophyll content, terpene synthesis etc.) are modified, gene expression is altered and growth reduced (stem elongation and dry weight) by exposure to EM radiation (Vian et al. 2016; Stefi et al. 2016, 2017a, 2017b, 2017c). These changes occur not only in the tissues directly exposed but also systemically in distant tissues. Although the long-term impact of these changes is rather unknown, it has to be a matter of speculation since the documents we have support the aspect that non-ionizing EM radiation cannot be considered as harmless. On the contrary, it proves to be a genuine environmental factor that readily evokes changes in plant metabolism (Vian et al. 2016).

Before coming to an end, we have to emphasize that, since **a)** ICNIRP (International Commission On Non-Ionizing Radiation Protection, an independent scientific organization responsible for providing advice on health hazard of non-ionizing radiation exposure) has set the SAR (Specific Absorption Rate) limit for the general public at 2 W per Kilogram (W/Kg) **b)** the EUROPEAN PARLIAMENT Directorate General for Research (European Parliament 2001) defined that Mobile phones must be low power radio devices transmitting and receiving microwave radiation at frequencies between 900 MHz and 1800 MHz, **c)** this type of radiation is considered as non-thermal, weak RF-EMF (Waldmann-Selsam et al. 2016; Halgamuge, 2017) and **d)** the cellular phone used (Motorola C115) has an FCC approval (09 June 2004 - FCC ID value: IHDT56EX1) with SAR 0.31 W/kg (head) or 0.44 W/kg (body) measured at 1800 MHZ, we may assume that the stress response observed in the leaves of *M. communis* has a causal relationship with RF-EMF and renders a strong evidence for the non-thermal origin of the RF-EMF effects.

5. Conclusions

Concluding, we may resume our data from young plants of *Arabidopsis thaliana*, *Gossypium hirsutum* and *Zea mays* grown under continuous exposure to non-ionizing radiation and the detrimental effects of this radiation on plant development, biomass production, photosynthetic pigments and chloroplast ultrastructure (Stefi et al. 2016, 2017a, 2017b, 2017c) along with the severe stress signs observed in radiated mature leaves of *Myrtus communis* individuals and contribute a huge "con" to the serious speculation on the effects of non-ionizing radiation to plant life.

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