



Original Research

The aftermath of long-term exposure to non-ionizing radiation on laboratory cultivated pine plants (*Pinus halepensis* M.)

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ABSTRACT

Sprouts of *Pinus halepensis* were incubated and cultured in the laboratory under controlled conditions to investigate their response to a long-term exposure to continuous, non-ionizing radiation emitted from the base unit of a cordless DECT system. Exposed plants, compared to their control counterparts, seem to be affected since they exhibit lower sprouting potential, minor fresh weight and biomass for both the above ground part and the root, reduction of their photosynthetic pigments and significantly increased ROS levels. Cotyledon, juvenile leaf, primary shoot and root structure seem similar in both control and exposed plants. What seems to be affected is the structure of chloroplasts in the exposed leaves. Many cells of the exposed leaves possess severely deformed chloroplasts with dilated or destructed thylakoid membranes although disruption of chloroplast envelopes was not observed.

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1. Introduction

Much consideration has been given and an extensive argument persists on the biological effects of electromagnetic (EM) radiation. Visible light, UV, LASER, X-rays and Gamma rays are EM radiations differing in frequency and, consequently, in energy (Kovács and Keresztes, 2002; Esnault et al., 2010; Abbas et al., 2017; Bibi et al., 2017). Ionizing radiation imposes living organisms to a series of alterations, usually leading to a biological injury (Sax, 1942; Jacobs, 1998; Gulston et al., 2002; Georgakilas et al., 2004; Prasad et al., 2004; Hardell and Carlberg, 2009; Yang et al., 2013). For instance, UV radiation seems to affect plant life in spite of the fact that plants, having the ability to exploit sunlight, do have some means of protecting the living cells from the radiation-induced damages of DNA (Hollósy, 2002). Gamma radiation, as well, has both stimulatory and inhibitory effects upon plant growth. These effects can be both morphological and ecological and they are reflected through species interactions at the community level (McCormick and Platt, 1962).

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). Therefore, we may assume that plants evolved learning to use these environmental signals, along with visible light in order to regulate their periodic functions. That means they may be sensitive to man-made RF fields (Haggerty, 2010).

Since the second half of the 20th century, wireless telephones and, recently, mobile phones turned to be the most common form of communication. This means that organisms living in the “civilized” world thrive within a “cloud” of radiations, mostly non-ionizing. The rapidly increasing use of the cellular technology resulted in an increase of electromagnetic radiations in the environment (Sharma and Parihar, 2014). 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 plants constitute an outstanding model to study the effect of High Frequency non-ionizing Electromagnetic Fields (HF-EMF) since their architecture (high surface area to volume ratio) optimizes their interaction with the environment (Vian et al., 2016), limited concern was given to plant reactions (Ledoigt, 2006; Roux et al., 2006; Pietruszewski et al., 2007; Haggerty, 2010; Kumar et al., 2015). Recently some data became available, on the biomass production, leaf anatomy and tissue organization, overall, for two widely used dicotyledons – the delicate, short-living, *Arabidopsis thaliana* (L.) Heynh. (Stefi et al., 2016) as a major experimental model plant and *Gossypium hirsutum* L. (upland cotton) as

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a widely cultivated crop plant of great economic importance (Stefi et al., 2017a) after their long term exposure to non-ionizing, DECT emitted radiation. *Zea mays* was also investigated lately and deformations of the thylakoid membranes in the agranal chloroplasts of the bundle sheath cells (C_4 chloroplasts) was reported, after long term exposure of laboratory cultivated plants to non-ionizing radiation (Stefi et al., 2017b).

The tree commonly known as Aleppo pine (*Pinus halepensis*), is a species native to the Mediterranean region. It grows wild from Morocco to East, in Greece, all over Malta and northern Tunisia and has a prominent population in Syria, Lebanon, southern Turkey, Jordan, Israel and the Palestinian territories. In Israel, it is called Jerusalem pine while, in Greece, the resin of this pine tree, amongst other uses, is used to flavor a Greek type of traditional wine named retsina. Having in mind that most lowlands in Greece are covered with pine trees, mainly the species *Pinus halepensis* (Mill.), and considering conifers (division: Pinophyta) as a plant group with major contribution to the global primary production, particularly in northern areas, we pondered on the impact of the EM radiation on the pine trees and we attempted, primarily, a literature search with interesting results.

Pine trees have previously been investigated for their response to various stressing factors. It is reported, for *P. halepensis*, that although Rubisco activity is reduced by 30% in the presence of ozone, no significant effect is noticed when plants were subjected to water stress (Pelloux et al., 2001). More data is given for *P. halepensis*, concerning the effect of air pollution on the nitrogen cycle (Wellburn et al., 1996), water stress on morphology, physiology and field performance (Royo et al., 2001), gamma-radiation on shoot viability through mitotic inhibition of the apical and sub-apical meristems (Donini, 1967) while serious effects of gamma (Brandenburg et al., 1962) and UV-B radiation (Laakso et al., 2000) were reported for other pine species. An interesting investigation reports the severe histological changes in the annual rings, the late wood, the number of the vertical resin ducts and the radial rays for two pine species in the proximal zone around the Chernobyl power plant (Skuterud et al., 1994).

Considering all the above we decided to probe the impact of the considered “innocent”, continuously emitted, non-ionizing, DECT EM radiation, on the young *P. halepensis* plants, investigating the effects on the cotyledons, leaves, shoots and roots.

2. Materials and methods

2.1. Plant material and exposure setup

Mature, new cones of *Pinus halepensis* Mill., were collected in early September 2016, from the “Ioulia and Alexandros N. Diomidis” Botanical Garden, West of Athens metropolitan area (38°00′34″ N, 23°38′43″ E, 309 m elevation). The cones were incubated at 120 °C for 2 min and then left for five days exposed to sunlight, during the day, for their scales to open. The seeds collected after scale opening were placed in Petri dishes for imbibition. Petri dishes were incubated in a ventilated, adjustable temperature P-Selecta incubator (Model No. 2000238–Barcelona, Spain) where they remained, for ten days, at 20 °C (70% humidity) until seeds sprouted. A build-in light source (Philips CorePro LED bulb, 11.5W=75W, at 2700 K, 105 mA), adjusted to a 16/8 light/dark cycle, provided 2500 Lux radiation (Photosynthetically Active Radiation=60 $\mu\text{mol m}^{-2} \text{s}^{-1}$) at the surface of the Petri dishes. After about twelve days, the eight cotyledons emerged and most seeds had grown to young seedlings (2n sporophytes). Selected plantlets were transferred and sown in 80 × 80 mm, 400 mL pots filled with wet perlite (amorphous volcanic glass formed by the hydration of obsidian), at pH 6.0. One young plant was sown in each

Table 1

Average and peak electrical field intensity, in each experimental setup, as measured for a 6-min period.

CAGE	Average	Maximum – integrated	Maximum – peak
Control	0.073 V/m	0.458 V/m	0.490 V/m
Exposed	2.072 V/m	11.320 V/m	27.460 V/m

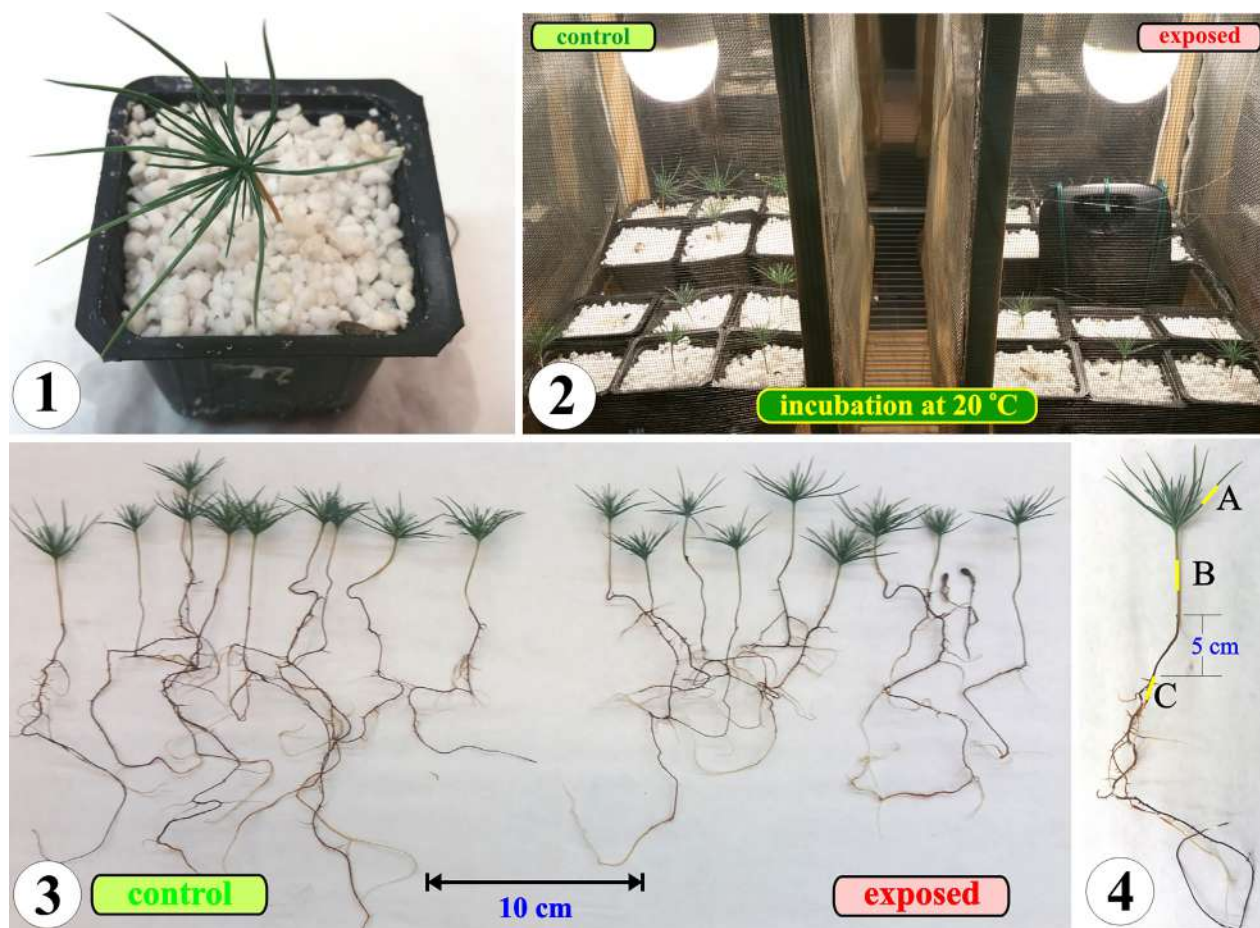
pot (Fig. 1). Twelve (12) pots were placed to fit in a transparent, rectangular 32 × 20 cm basin, where 20 mL of hydroponic nutrient solution (Hoagland) was added. Then the pot-containing basins were accommodated one in each of the two Faraday cages (40 × 40 × 25 cm, covered with 0.8 mm mesh – 0.1 mm stainless steel wire) (Fig. 2). After their construction, these cages were thoroughly tested for their ability to prevent any leakage of radiation emitted from within, to the environment. The light source (Philips CorePro LED bulb, 11.5 W = 75 W, at 2700 K, 105 mA), mounted on the ceiling of each cage, produced 2500 lux radiation (Photosynthetically Active Radiation = 60 $\mu\text{mol m}^{-2} \text{s}^{-1}$) at the surface of the pots.

A ventilated, adjustable temperature incubator (P-Selecta) was used and the two cages were accommodated in it, side by side. The plantlets remained there for 50 days, at 20 °C. The cage on the right hosted the base unit of a Digital Enhanced Cordless Technology (DECT) telephone (General, Model 123) (Fig. 2). The DECT base unit was positioned in the cage in the middle of the two groups of pots (Fig. 2) and remained there in a 24 h a day, 7 days a week, pulsed transmission mode, at 1882 MHz, as described by Margaritis et al. (2014), while the light/dark programme of the chamber was adjusted to a 16/8 cycle (Stefi et al., 2016, 2017a, 2017b). Pots were watered every 5 days with 100 mL tap water. Incubator temperature was set at 20 °C since this was reported to be the proper temperature for optimal germination and growth rate of *P. halepensis* (Thanos and Skordilis, 1987).

Radiation was measured within each one of the two cages while the DECT unit was in transmission mode within the right cage (Fig. 2). A NARDA SRM3000 (Germany) spectrum analyzer was used for these measurements. The corresponding electrical field intensity (average and peak), in each experimental setup, was measured for a 6-min period according to ICNIRP (1998) guidelines as in Table 1. For cross-checking the above measurements, a broadband field meter was used (TES-92, 50 MHz – 3.5 GHz, Electromagnetic radiation detector – TES Electrical Electronic Corp. Taipei, Taiwan, R.O.C.). Although this instrument is of lower precision, radiation was, once more, measured at the value of 490.1 mV/m within the control cage (Fig. 2–left cage) while it reached the value of 27.46 V/m (27.460 mV/m, at 1882 MHz) (55 fold higher) in the cage of the exposed plants (Fig. 2–right cage).

2.2. Microscopy

At the end of the experimental period, the pots were removed from the cages and smashed in water to acquire the plants which were washed to remove any remnants of the culturing substrate from the roots and placed on a filter paper (Fig. 3). The fresh weight of the plants in each group was measured before the plants were incubated for drying, at 60 °C, for three days. Then the dry weight (biomass) was measured. In all plants the fresh weight and the dry biomass were weighed for their above ground part and their root system. A small part from the centre of one cotyledon from each plant (yellow bar A in Fig. 4), one leaf from each plant, a part from the middle of the stem (yellow bar B in Fig. 4) and a part of the root, 5 cm below the shoot (yellow bar C in Fig. 4) from each plant, were removed in random, sectioned in to small (2 mm length) pieces and fixed in phosphate buffered 3% glutaraldehyde (pH 6.8) at 0 °C for 2 h. Some of the pieces, from each organ, were dehydrated in



Figs. 1–4. Culture setup. 1) A pot, filled with wet perlite and a young plant of *P. halepensis*, before the start of the experiment. 2) The pots accommodated in the Faraday cages. The DECT base unit appears in the right cage. 3) The yield after the end of the experiment. 4) A young plant marked at the axis areas where pieces were cut for fixation. A, from the cotyledon, B from the middle of the stem and C from the root, 5 cm below the transition area.

graded acetone series, critical point dried, coated with gold and viewed with a JEOL JSM-6360 Scanning Electron Microscope

The rest of the tissue was post fixed in 1% osmium tetroxide in phosphate buffer, dehydrated in graded ethanol series and embedded in Durcupan ACM (Fluka, Steinheim, Switzerland). Semithin sections were cut with an LKB Ultratome III. They were placed on glass slides, stained with 0.5 toluidine blue O (in 1% borax solution) as a general stain and observed with an OLYMPUS CX41 (Japan) light microscope. Ultra thin sections were placed on 100 mesh, copper grids, double stained with lead citrate and uranyl acetate (Reynolds, 1963) and viewed with a Phillips EM-300 Transmission Electron Microscope (TEM), at 80 kV. Literature for double fixation and staining for TEM is cited in detail by Christodoulakis et al. (2009) and Christodoulakis et al. (2010).

2.3. Pigments protocol

Chlorophyll pigments were extracted with 1 mL 80% acetone, overnight, at 4 °C from a small quantity of leaf tissue, equal for both leaf types (about 50 mg). The supernatant was transferred to a 1-mL glass cuvette and measured in UV/Vis Specol photometer (Zeiss). Absorbance was recorded from 400 to 700 nm. We focused at both 663.6 and 646.6 nm, wavelengths corresponding to chlorophyll *a* and chlorophyll *b* respectively. We also checked for the absorbance of chlorophyll *c*, at 625 nm. Quantification of pigment content was calculated using molar extinction coefficients specifically for this method (Gechev et al., 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

formula used to calculate the total chlorophyll content and normalize per fresh weight was: $\text{Chlorophyll } a + b = 19,54A_{646.6} + 8,29A_{663.6}$. For beta carotenes and xanthophylls, where no formula is available for quantification, the absorbance value was solely utilized. Exactly the same protocol was used for extracting the photosynthetic pigments from the cotyledons.

2.4. Total ROS estimation

ROS levels were measured using 10 mM of the oxidant sensitive fluorescent acetyl ester CM-H₂DCFDA (5-(and-6)-chloromethyl-2',7'-dichloro-dihydro-fluorescein diacetate) dissolved in DMSO. CM-H₂DCFDA 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 12,000g 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-H₂DCFDA diluted in mM Tris-HCl at 37 °C in the incubator. Then, the ester was removed and this was followed by 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. Plant morphology and biomass

Although the initial impression, after the first visual comparison, was that the plants from the two groups were similarly shaped and, more or less, equally developed, a careful approach reveals that the exposed group appears to be significantly retarded in development (Fig. 3).

The real difference of the yield between the two groups is clearly demonstrated through weighing the fresh and the dry mass (biomass), in both groups. Comparing the shoots (above ground part = stem and leaves) (Fig. 5) it seems that both fresh weight and biomass of the shoots of the exposed plants are reduced to about 75% and 60% of the initial weight respectively while the roots exhibit more pronounced reduction for the exposed plants which reaches 60% of the initial fresh weight and 50% of the initial biomass (Fig. 6).

3.2. Pigment content and reactive oxygen species

Comparing the control plants to their exposed counterparts, in relation to the photosynthetic pigments of their cotyledons (Fig. 7), we may assume that no evident reduction is caused. Yet, concerning the leaves of the exposed plants, a significant reduction of these pigments was measured (Fig. 8).

An impressive part of this experiment has to do with the differences of the ROS values concerning the cotyledons, leaves, stems and roots of the control and the exposed plants (Fig. 9). Comparing the values between control and exposed organs we point out the substantial increase of the ROS in the exposed plants. For the leaves the increase rises up to about 100%, for the cotyledons up to 160%, for the shoots 90% while for the roots a lower increase of 65% was measured.

3.3. Light microscopy

Light microscopic observations were carried out in cross sections of cotyledons, leaves, stems and roots from control and exposed plants. Numerous observations revealed (Figs. 10 and 11) that the cotyledons of the control plants appear a slightly more developed. The cross sections of these cotyledons are rather trapezoid while the sections of the cotyledons of the exposed plants appear always triangular in cross sections. No differences in the arrangement and development of the conductive tissue could be observed while the stomata of the exposed cotyledons (Fig. 13) appear identical in size and structure with those of the cotyledons from the control plants (Fig. 12). What seems very interesting with the cotyledons, from both plant types, is the appearance of some polymorphonuclear cells in the parenchymatous tissue at the abaxial side of the cotyledons. These cells appear rarely and have an irregular shape, not the common shape of the photosynthetic cells of the pine needles (Figs. 14 and 15).

Cross sections of pine needles (leaves) were also investigated. Epidermal cells and stomata, photosynthetic cells, conductive tissue and resin ducts were carefully inspected.

Leaves of both plant groups appear not fully developed. Their conductive tissue is under construction (yellow arrow in Figs. 16 and 17) while the endodermal cells round the bundle still

have their primary walls. Conductive tissue seems to grow unaffected in the needles of the exposed plants (yellow arrow in Fig. 23). It is interesting to point out that the so typical, for the pine leaves, lobed mesophyll cells are not fully developed yet. Most of the mesophyll cells, below the epidermal tissue, appear having a palisade shape while other cells, close to the conductive bundle, appear lobed (Figs. 16 and 20). Young resin ducts (Figs. 18 and 22) appear to carry on their schizogenous development normally, in contact to the epidermal cells. Some of them (Fig. 19) are close to maturity. Stomata are sunken and appear to be below the level of epidermal cells (Figs. 17, 19 and 21) with the hypostomatal space gaping under the guard cells. Stomata of the exposed needles appear intact (Fig. 21). Condensed, intensely stained particles of heterochromatin can be observed within their nuclei (Fig. 21). Photosynthetic cells soon acquire their particular lobed shape and start accumulating osmiophilic secondary metabolites (Figs. 16, 18 and 20, 22).

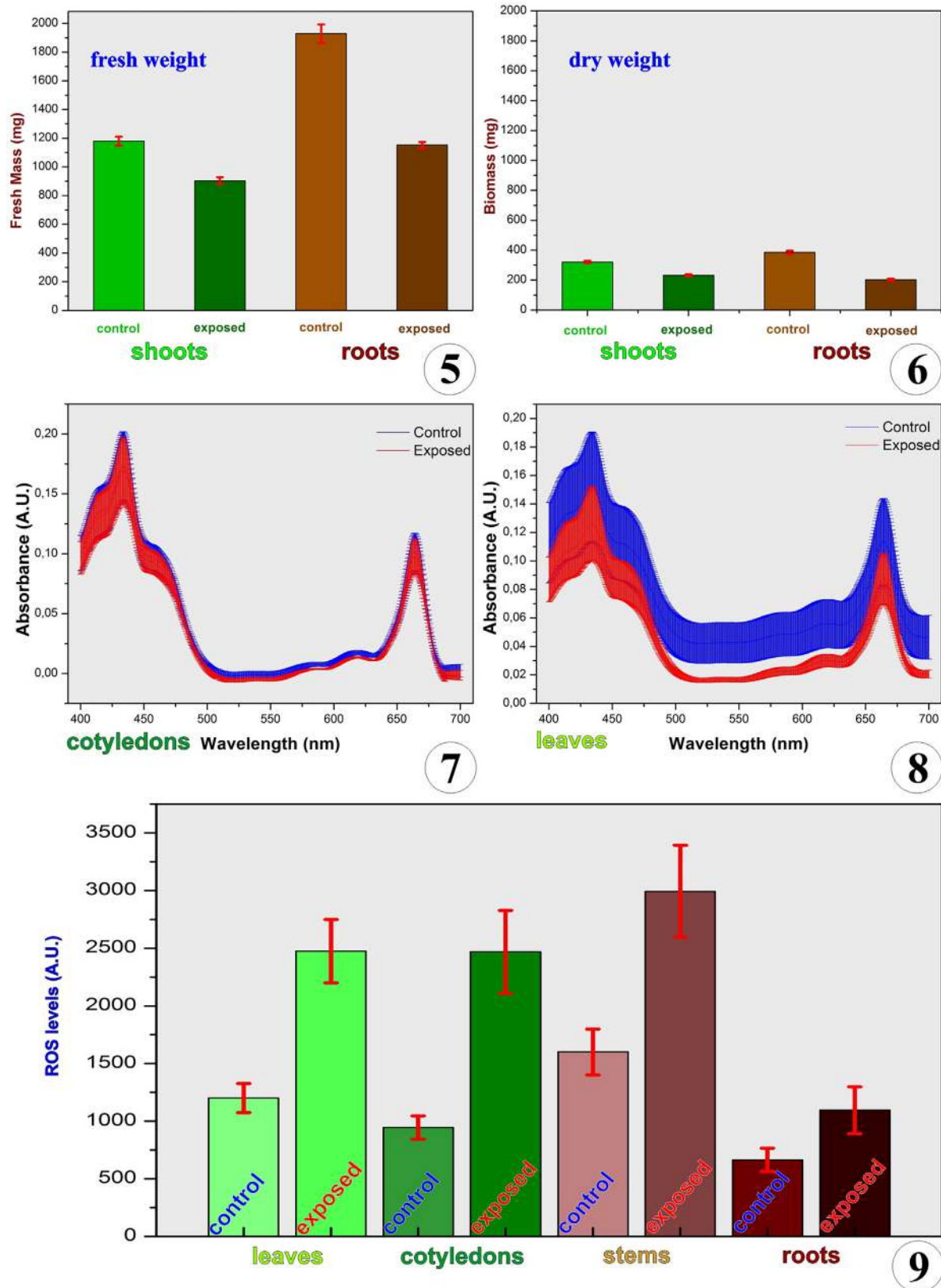
The stems, in the plants of both groups, appear identical (Figs. 24 and 27). Special attention was given to the cambial activity (pink arrows in Figs. 24, 25 and 27, 29) and the development of xylem as well as to the arrangement and development of the resin ducts (yellow circles in Figs. 24, 25, 26 and 27, 29). Five or six resin ducts appear to develop between the xylem and just below the active cambium (yellow circles in Figs. 24, 25, and 27, 29). The only difference observed is that the epithelial cells of resin ducts in the stem of the exposed plants appear to excrete resin in the lumen of the duct (green arrow in Fig. 30), something we never observed in the ducts of the control plants (Figs. 25 and 26). Epidermal cells present, from this developmental stage, a very thick, secondary wall (Fig. 28) while stomata still appear on the epidermis (red circles in Figs. 24 and 27) (Fig. 28). Only a few cells of the stem parenchyma accumulate secondary metabolites (Figs. 24 and 27).

Concerning the roots, cross sections indicate the prompt development of the secondary xylem elements among which the resin ducts develop (yellow circles in Figs. 31 and 34). Initially the roots appear with four rays while, later in root development, these rays may turn to five or six with the xylem poles embedded between resin ducts (Fig. 32). Cambial activity is prominent (pink arrows in Figs. 31, 33 and 34, 35). A lining of cortex cells accumulating secondary metabolites, thus appearing osmiophilic, can be observed in the roots of both plant groups (Figs. 31 and 34). The resin ducts appear developed (Figs. 33 and 36) and no structural differences can be observed between the two groups.

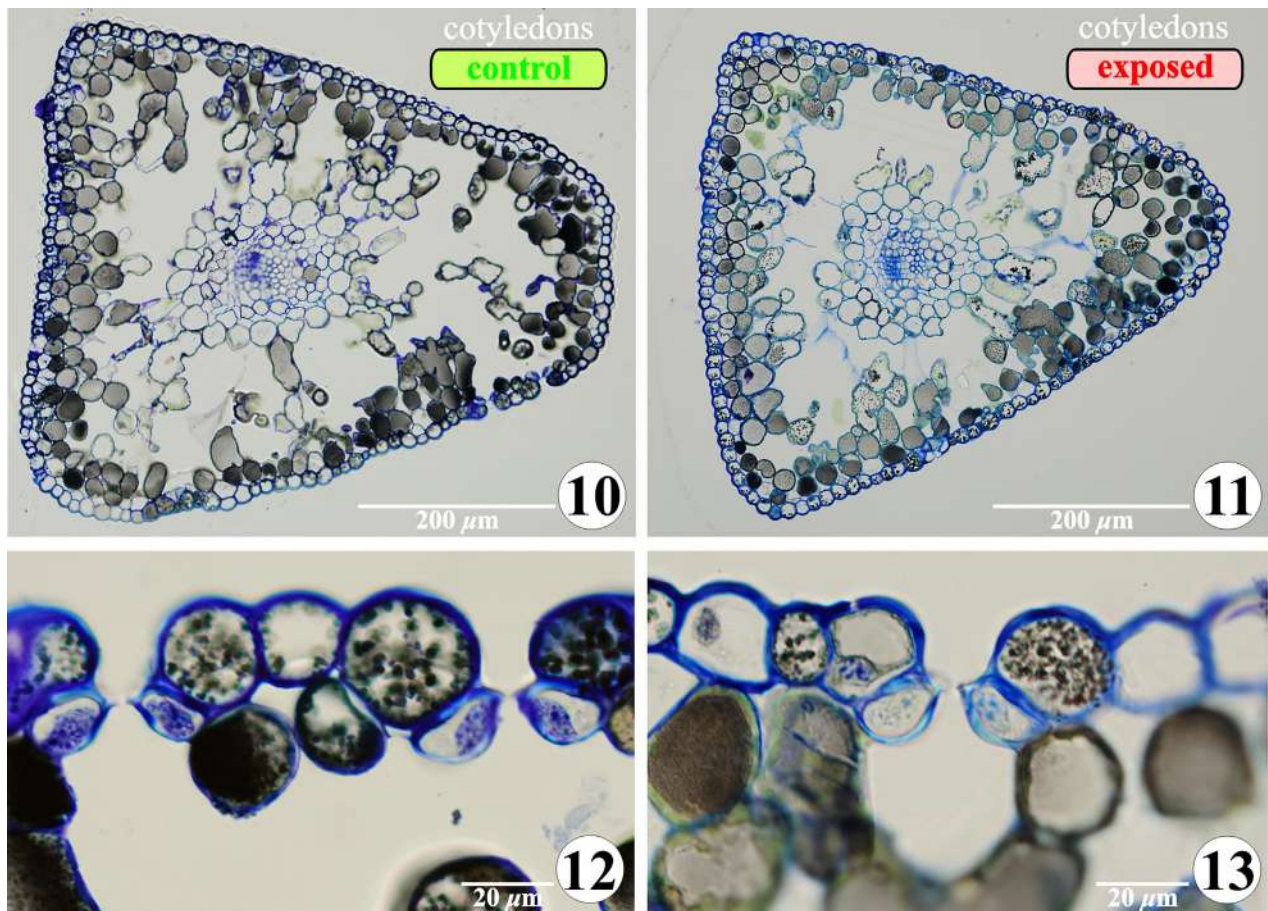
3.4. Scanning electron microscopy (SEM)

Scanning Electron Microscopy (SEM) was employed to depict the impact of radiation on the external morphology, i.e. the epidermal cells, stomatal arrangement and frequency and guard cell structure of the cotyledons and leaves of the two plant groups. Cotyledons of the exposed plants (Fig. 37) appear identical to those of their control counterparts. Stomata do not seem to be affected (Fig. 38) although some of them appear elevated instead of being located at the bottom of the stomatal pit (Fig. 39). Stomatal frequency cannot be counted in the conventional way (stomata/ mm^2) since stomata appear in rows which are not evenly scattered. Stomata were counted per mm of length. The values for the control (45 ± 8) and the exposed cotyledons (43 ± 7) are very close.

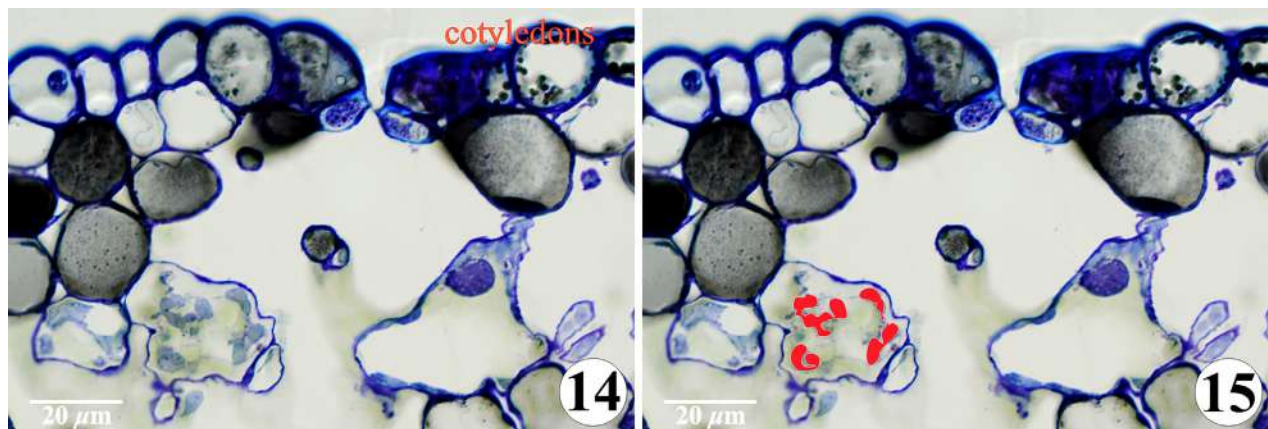
Pine needles also appear identical in both leaf types. Epidermal cells and the marginal teeth (Figs. 40 and 41) appear unaffected. Sunken stomata and the opening between the guard cells and the edge of the stomatal fissure, appear identical in both leaf types (Figs. 42 and 43). Stomata were counted for the control (30 ± 8) and the exposed leaves (34 ± 7) per mm of needle length. Leaves seem to possess fewer stomata than cotyledons.



Figs. 5–9. Depicting weight, pigment and ROS values. 5) Fresh weight for shoots and roots. 6) Dry weight for shoots and roots. 7) Absorbance of the photosynthetic pigments by the cotyledons. 8) Absorbance of the photosynthetic pigments by the leaves. 9) ROS levels for all the plant parts. Light colours for the control and dark colours for the exposed plants.



Figs. 10–13. Light Microscope – cotyledons. 10) Cross section of a cotyledon from a control plant. 11) Cross section of a cotyledon from an exposed plant. 12) Detail of a cross sectioned cotyledon (control). Notice the two sunken stomata, their guard cells and the nucleus within each one of them. 13) Detail of a cross sectioned exposed cotyledon. Epidermal cells, one stoma and the nucleus of the guard cell, at the right, can be observed.

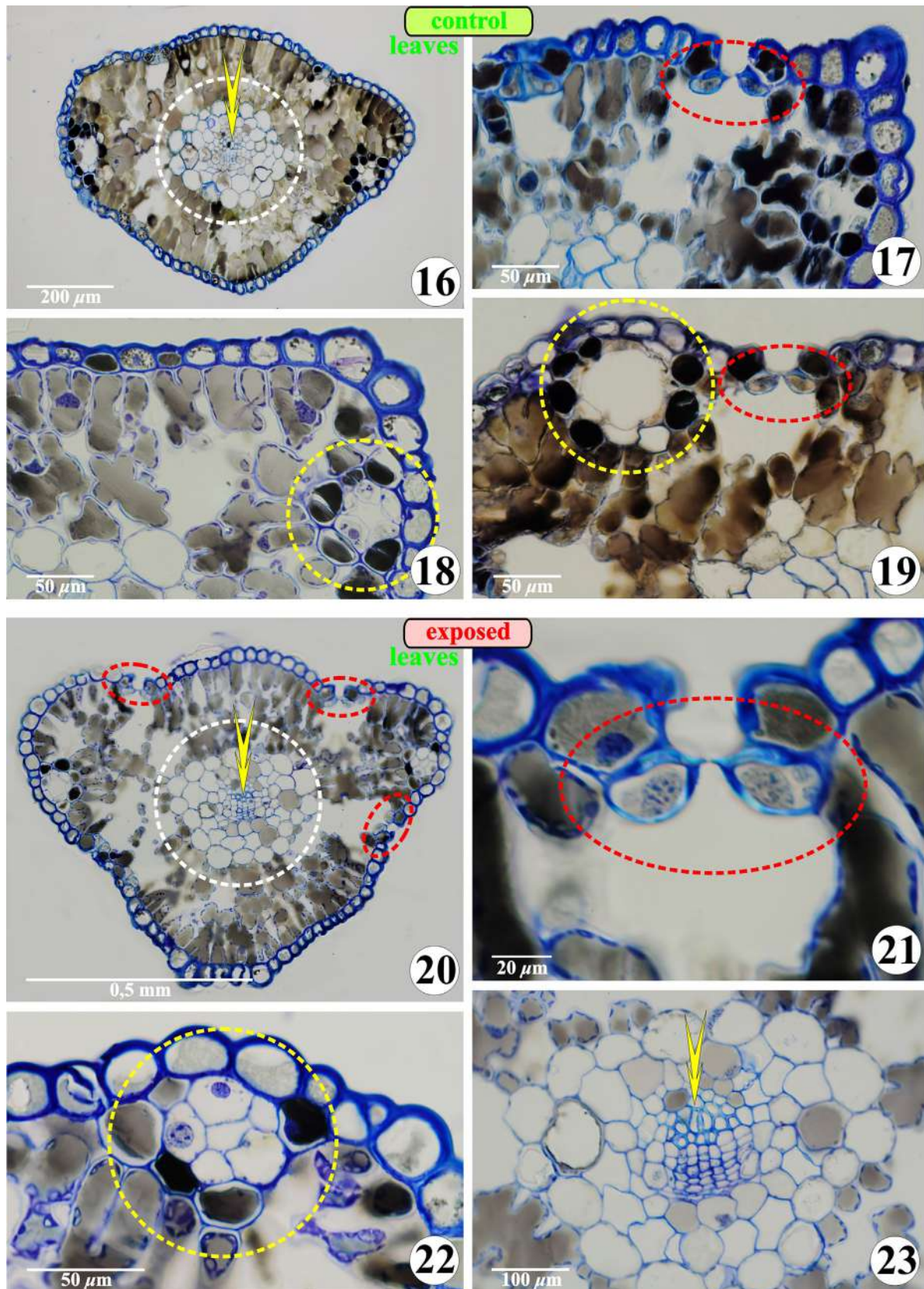


Figs. 14–15. Light Microscope – cotyledons. 14) An interesting observation in many cells of the cotyledons, mainly those accommodated close to the epidermal tissue, is the lobed nucleus. 15) Exactly the same figure as Fig. 14 with the multi-lobed nucleus marked with red colour. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

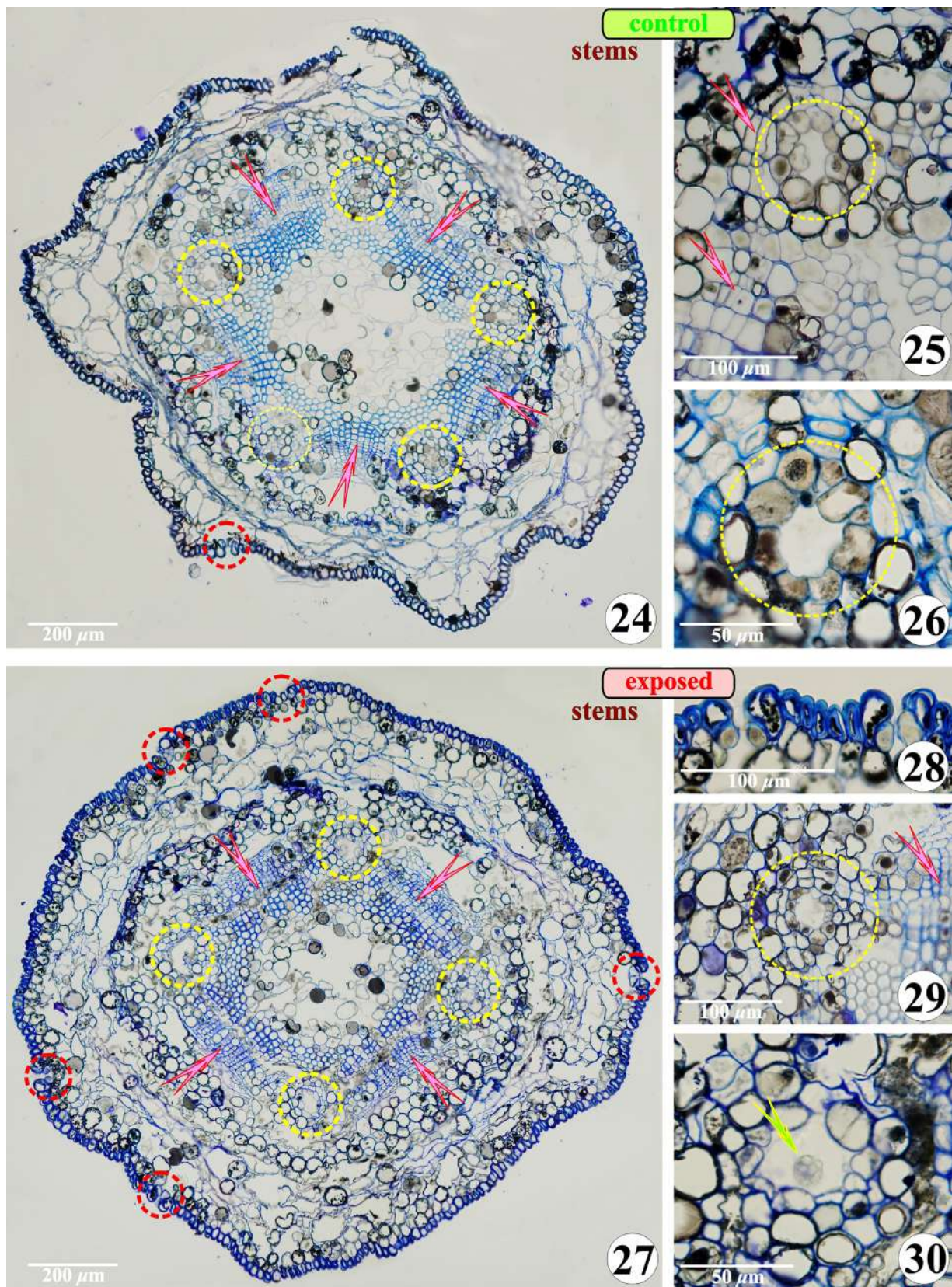
3.5. Transmission electron microscopy (TEM)

Observations of ultra-thin sections from pine needles revealed the detailed structure of the epidermal tissue with the supporting lining of lobed cells, the inner photosynthetic cells (Fig. 44) and, sometimes, the resin ducts (Fig. 45). The cells of these tissues, excluding palisade shaped cells, do not appear to have any deformations or damages, when control and exposed leaves are compared

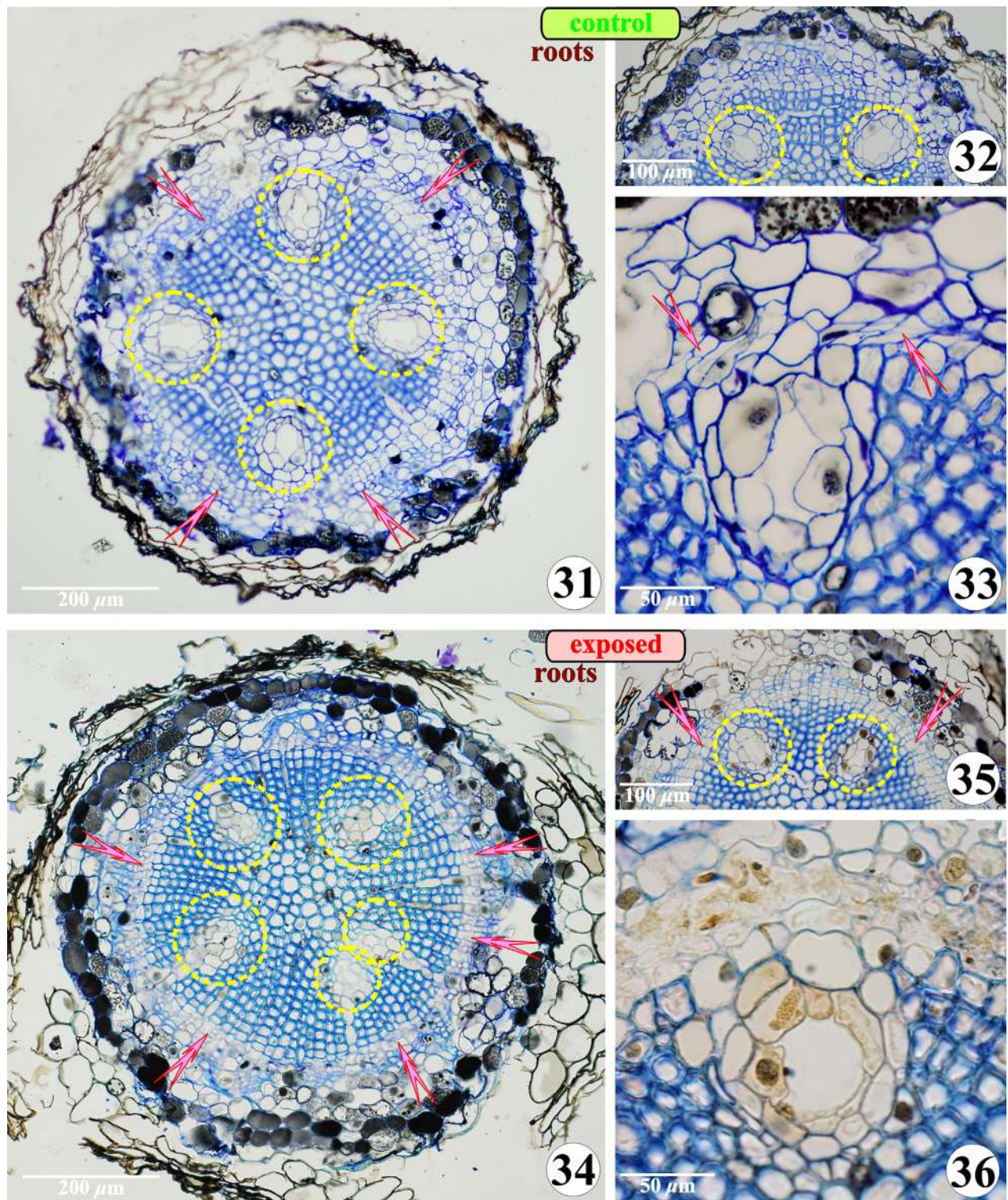
at the ultrastructural level. Chloroplasts, of both leaf types, exhibit a round or lens-shaped structure, dense stroma, typical arrangement of their well elaborated fretwork and multilayered grana. It is interesting to point out that chloroplasts, of both control (white arrows in Fig. 46) and exposed leaves (white arrows in Fig. 47), sporadically appear to have slightly swollen membranes. Mitochondria appear typical in both leaf types (light green arrows in Figs. 46 and 47).



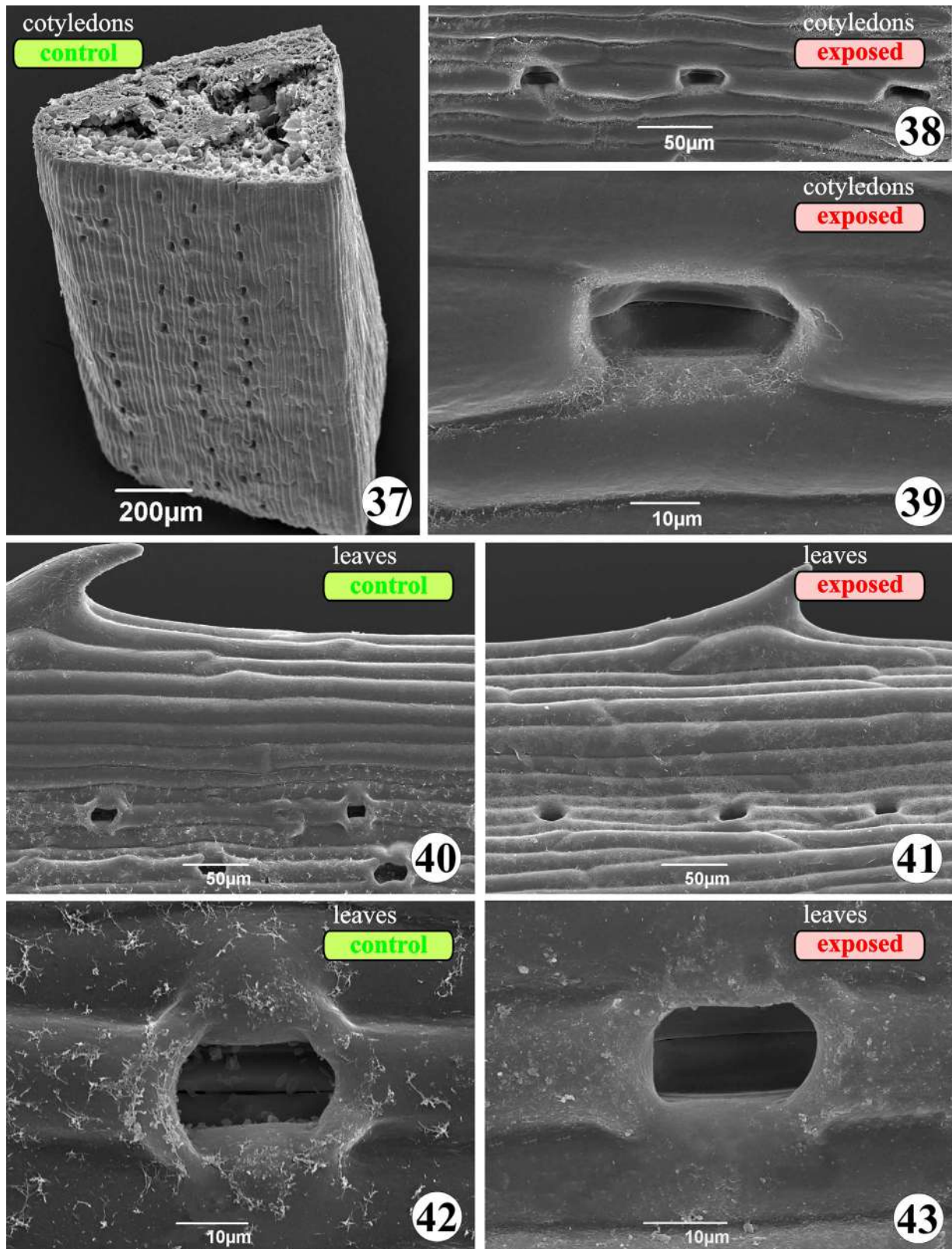
Figs. 16–23. Light Microscope – leaves (pine needles). 16) Cross section of a pine needle from a control plant. Note the conductive bundle, in the middle, the large, premature endodermal cells surrounding it, the dark, osmiophilic content of the mesophyll cells and the single layered epidermis. 17) Cross section of a needle (control plant) with a sunken stoma. 18) Higher magnification of a cross section from a needle of a control plant. One stoma (red outline) and one resin duct (yellow outline) can be observed. 20) Cross section of a pine needle from an exposed plant. The conductive bundle, in the middle, the large, premature endodermal cells surrounding it, the dark, osmiophilic content of the mesophyll cells and the single layered epidermis, can be observed. Three stomata are marked with red outline. 21) Cross section of a needle (exposed plant) with a submerged stoma. Note the nuclei with condensed chromatin. 22) Larger magnification of a cross section from a needle of an exposed plant. A well shaped resin duct (yellow outline) can be observed. 23) The conductive tissue of an exposed needle. Cells seem normally arranged with no deformations of the conductive elements (group of cells in the middle – yellow arrow). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)



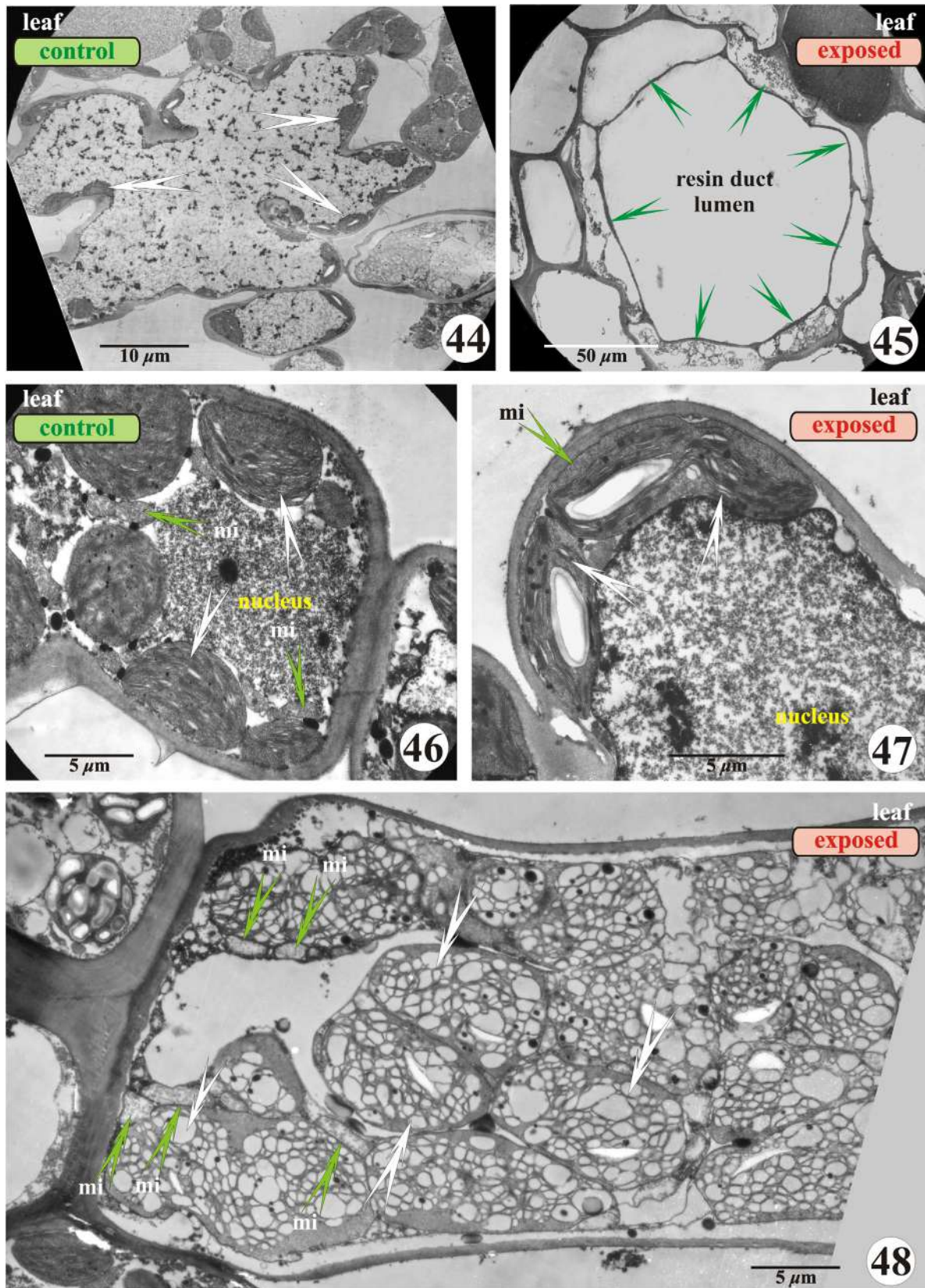
Figs. 24–30. 2nd experiment. Light Microscope – pine stems. 24) Cross section of a pine stem from a control plant. Note the epidermal tissue, one stoma (red circle), five resin ducts (yellow circles) and the confined areas of cambial activity (pink arrows). 25) Detail of a cross section of a stem (control plant) with dividing cambial cells (pink arrows) and a newly formed resin duct. 26) Cross section of a resin duct (control plant). Note the darkly stained epithelial cells accumulating various metabolites. 27) Cross section of a pine stem from an exposed plant. Epidermal tissue, stomata (red circles), four resin ducts (yellow circles) and the areas of cambial activity (pink arrows) seem undisturbed. 28) Detail of a cross section from the stem of an exposed plant showing stomata. 29) A young resin duct and dividing cambial cells (pink arrow). 30) Premature resin duct with signs of secreting activity. The green arrow points at a droplet of resin. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)



Figs. 31–36. Light Microscope – pine roots. 31) Cross section of a pine root from a control plant. Note the deformed epidermal tissue, the resin ducts (yellow circles) and the areas of cambial activity (pink arrows). 32) Detail of a cross section of a root (control plant) with the area of cambial cells between two young resin ducts. 33) A young resin duct with the epithelial cells before the initiation of their secreting activity. 34) Cross section of a pine root from an exposed plant. Resin ducts (yellow circles) and the cambial activity (pink arrows) seem undisturbed. 35) Detail of a cross section from a root of an exposed plant with the area of cambial activity (pink arrows) and two young resin ducts (yellow circles). 36) A young resin duct surrounded by xylem and cambial cells. Epithelial cells do not present any secreting activity. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)



Figs. 37–43. Scanning electron micrographs. 37) A portion of a cotyledon from a control plant. Stomata appear in rows along the axis of the cotyledon. 38) Detail from the surface of an exposed cotyledon. Three stomata appear in a row. 39) The surface of an exposed cotyledon. The stoma appears undisturbed. 40) The surface of a control leaf presenting the epidermal cells, a marginal hair and the stomata. 41) The surface of an exposed leaf presenting the epidermal cells, a marginal hair and the stomata. The whole structure appears undisturbed. 42) A sunken stoma from a control leaf. 43) A sunken stoma from an exposed leaf.



Figs. 44–48. Transmission electron micrographs. 44) Low magnification of a typical lobed cell from the mesophyll of a control leaf. White arrows point at some of the chloroplasts. 45) Low magnification of a resin duct from an exposed leaf. Secreting activity cannot be traced. The structure seems undisturbed. 46) A mesophyll cell from the leaf of a control plant. White arrows point at the chloroplasts while green arrows point at the mitochondria. 47) A mesophyll cell from a leaf of an exposed plant. White arrows point at the chloroplasts that seem to accumulate starch. Green arrows point at the mitochondria. 48) A mesophyll cell from a needle of an exposed plant. Numerous cells of this kind present totally disintegrated chloroplasts (white arrows) and rather deformed, granular and electron opaque mitochondria (green arrows). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Many of the lobed, sub-epidermal photosynthetic cells of the exposed tissue exhibit chloroplasts with a strange, significantly disturbed or wrecked network (white arrows Fig. 48). They possess granulated but not dense stroma without any grana. They seem to suffer an extended swelling of their thylakoids while, sometimes, exhibit osmiophilic granules within these swollen membranes (Fig. 48). Thylakoid dilation and swelling affects not only peripheral but also their internal thylakoids. Disruption of the chloroplast envelope was not observed. Mitochondria seem to be totally wrecked since they have no cristae at all (light green arrows in Fig. 48). Plasmalemma of these cells is not affected as well.

4. Discussion

Recent investigations revealed that the reduction of fresh weight and biomass is something common in plants suffering long term exposure to non-ionizing radiation (Sharma et al., 2009; Kumar et al., 2015; Stefi et al., 2016, 2017a, 2017b). The same seems to apply to *P. halepensis* as well, since the fresh weight and, consequently, the biomass are strongly reduced in both the shoots and the roots of the exposed plants. This reduction seems to be higher for the root than for the above the ground part. We may assume, after all, that plants investigated so far, present, when exposed to non-ionizing radiation, retarded growth, which means inferior primary production.

Photosynthetic pigments of the cotyledons do not seem to be affected in the sprouts of the exposed plants yet they are strongly reduced in the new leaves of these plants (exposed). This is probably an effect of the stress conditions experienced by the exposed plants, indicated by the significant increase of ROS levels in both the leaves and the cotyledons of these plants (Fig. 9). It has been reported that, in *Vigna variata* (Sharma et al., 2009), the same stress may break down the photosynthetic pigments or cause the destruction of many of the chloroplasts. ROS are produced normally through the plant's cellular metabolism and, despite their destructive activity; they are also used as second messengers in a variety of cellular processes (Sharma et al., 2012). Yet, various environmental stresses leading to excessive production of ROS, result in oxidative damage and, ultimately, in cell death (Sharma et al., 2012). It has recently been confirmed that ROS production can also be induced by exogenous factors such as high temperature or UV radiation (Manta et al., 2014) and can cause, at the cellular level, a wide range of responses, from proliferation to cell death (Manta et al., 2014). Anyway, the reduction of the photosynthetic pigments, whatever the cause is, results in inferior photosynthetic activity for the exposed plants and, consequently, less primary productivity (less biomass).

Cotyledon structure is hardly affected. This seems prospective since cotyledons follow the development of the embryo and remain dormant until seed imbibition and germination (Fahn, 1990). Therefore, they appear as prefabricated structures which do not alter after sprouting, under the specific conditions of exposure to radiation. The presence of polymorphic nuclei observed might be due to polyploidy which has been reported for the cotyledons of many species (*Phaseolus*, *Pisum*) although *Pinus* species are so far exempted from literature.

Needle (leaf) structure, concerning epidermis and stomata (Stabentheiner et al., 2004), photosynthetic tissue arrangement and the resin duct development, do not seem to be affected. This also seems normal since, to the best of our knowledge, there are only two investigations for conifer leaf deformations under environmental stress conditions. The first one reports deformations of the xylem elements of the pine needles under water stress (Cochard et al., 2004). The other one reports deformations of the conductive elements (tracheids) in the leaves of a tropical conifer (*Podocarpus grayi*) under water stress (Brodrick and Holbrook, 2005).

Stomatal structure, settlement and frequency as well as the shape of the epidermal cells and their arrangement, seem more or less uniform in both types of cotyledons and leaves. Stomata appear always inferior in number on the exposed leaves yet this difference cannot be evaluated since it falls within the limits of the statistical error.

Stem structure and cambial activity seem not to be affected either. Although we have numerous reports on the effects of environmental factors on the structure of the stem of the dicotyledons, only a few are available for the pine stem (Quirk and Freese, 1976; Antonova and Stasova, 1993; Wodzicki, 2001). It also seems probable that in the primary stages of stem growth, as those we observed in *P. halepensis*, cambial activity cannot give any indications of environmental impact, although environmental factors are those who regulate cambial activity on a long-term basis.

Resin ducts seem to initiate their secreting activity sooner in the exposed plant stems. Since stressing conditions induce the secretion of secondary metabolites in most Mediterranean plants (Christodoulakis, 1989) and resin flow is reported to be differentially affected by environmental conditions (Lombardiero et al., 2000), then premature or increased resin excretion indicates that the plant sustains stressing conditions.

Root structure, root cambial activity and resin duct development seem not to be affected in the exposed plants. Although the changes on this organ's morphology or tissue arrangement are either minimal or cannot be observed, the dry weight of the exposed roots is significantly lower compared to that of their control counterparts (Figs. 5 and 6). This controversy, gives rise to a challenging question as to how far and in what way does the soil, in various depths, act as a barrier to radiation.

Our observations indicate that chloroplast structure is severely affected in many cells of the exposed leaves. It has been well documented that chloroplasts are cellular organelles vulnerable to various environmental stresses (Wellburn et al., 1972; Holopainen and Nygren, 1989; Ladanova, 1993; Pritchard et al., 1997). In *Phlomis fruticosa* individuals, exposed to high levels of air pollution, the leaves possess chloroplasts with displaced or dilated membranes and swollen grana which, finally, burst resulting to the lysis of the organelles (Psaras and Christodoulakis, 1987). In the leaves of *Pinus monophylla*, the chloroplasts change their shape from ellipsoid to irregular and possess dark stained plastoglobuli when exposed to the stressing conditions such as acid rain (Back and Huttunen, 1992). Although ultrastructure of the coniferous plants has not been studied in detail, a few reports (Back and Huttunen, 1992; Boddi et al., 2002; Grill et al., 2004; Stabentheiner et al., 2004) provide some information on the gymnosperm chloroplast structure. It has been observed that some *Pinus* chloroplasts, probably at the stage of senescence, are full of small vesicles (Whatley, 1975). In the case of the current investigation, chloroplasts are actually far from being considered as senescing since only juvenile leaves have been used.

It has been reported that chloroplasts produce, during cellular stress, reactive oxygen species (ROS) which are known to act as regulators of programmed cell death (PCD) in plant and animal cells (Doyle et al., 2010). Later, it was spelled out that, inside the chloroplasts, several abiotic stresses (including drought, high light, salinity, or extreme temperatures) induce a reduction in CO₂ assimilation rates with a consequent increase in reactive oxygen species (ROS) production, ultimately leading to leaf senescence and yield loss. Photo-oxidation processes should therefore be mitigated to prevent leaf senescence (Pintó-Marijuan and Munné-Bosch, 2014). Although plants have evolved several mechanisms to either prevent the formation of ROS or eliminate them (Pintó-Marijuan and Munné-Bosch, 2014), in our case of *P. halepensis*, these mechanisms cannot compensate the significant increase in ROS, probably

because the plants were exposed to excessively stressing conditions.

5. Conclusions

Young plants exposed to non-ionizing radiation seem to be structurally unaffected but present severe deformations and damages at the ultra structural level, especially in concern to their chloroplasts. The reduction of photosynthetic pigments causes reduced photosynthesis and, eventually, lower primary productivity which is a heavy “mortgage” for their future life that does not seem thriving.

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