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Anatomy, photochemical activity, and DNA polymorphism in leaves of dwarf tomato irradiated with X-rays

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Abstract

The response of higher plants to ionising radiation depends on factors related to both radiation properties and plant features including species, cultivar, age, and structural complexity of the target organ. Adult plants of dwarf tomato were irradiated with different doses of X-rays to investigate possible variations in leaf morpho-anatomical traits, photosynthetic efficiency, and genomic DNA. In order to assess if and how responses depend on leaf developmental stage, we analysed two types of leaves; nearly mature leaves (L1) and actively developing leaves (L2), whose lamina size corresponded to 70 and 25 %, respectively, of the lamina size of the fully expanded leaves. The results show that the X-rays prevented full lamina expansion of the L2 leaves at all doses and induced early death of tissue of plants irradiated with doses higher than 20 Gy. Most anatomical modifications were not clearly dose-dependent and the radiation-induced increase in phenolic compounds was irrespective of dose. At high doses of X-rays (50 and 100 Gy), photochemical efficiency decreased significantly in both leaf types, whereas total chlorophyll content significantly decreased only in the L2 leaves. The random amplification of polymorphic DNA data show that the X-rays induced mutagenic effects in the L2 leaves even at low doses despite the absence of severe phenotypic alterations. Genetic structure found in the population of samples corroborates the results of anatomical and eco-physiological analyses: the 20 Gy dose seems to mark the threshold dose above which genetic alterations, structural anomalies, and perturbations in the photosynthetic apparatus become significant, especially in the actively expanding leaves.

Additional key words: chlorophyll content, leaf development, photosystem 2, RAPD.

Introduction

Ionising radiation alters plant growth and development by inducing genetic, physiological, and morphological changes (De Micco *et al*. 2011, Arena *et al*. 2014a). Knowing plant response to different doses of ionising radiation is useful for ecological purposes and in space-oriented researches aiming to select radioresistant species for cultivation in extra-terrestrial outposts (De Micco *et al*. 2014b). However, unequivocal plant responses to radiation have not been traced yet because of the variability of results obtained in experiments where different types of radiation have been applied to different target organs/organisms to address specific endpoints.

At present, most available information derives from experiments in which dry/wet seeds or seedlings were exposed to different types of ionising radiation (Shi *et al.* 2010), whereas leaves, roots, or other parts of adult plants were infrequently exposed as target organs (Tanaka *et al.* 2002, Yang *et al*. 2007, Arena *et al.* 2013, 2014a, De Micco *et al.* 2014a). Even if plants can survive radiation doses that are lethal for animals, some structural

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Abbreviations: F_v/F_m - maximum efficiency of PS 2 photochemistry; Φ_{PS2} - quantum yield of PS 2 linear electron transport; L1 - nearly mature leaves, L2 - developing leaves; LA - leaf area; LT - lamina thickness; OPB - Operon Primer B; PS - photosystem; TLA - theoretical lamina expansion.
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modifications have been evidenced in plants as a response to high doses of radiation (Arena *et al.* 2014b). The responses of a specific plant or organ to high doses of ionising radiation include alterations in the cell cycle and plant metabolism leading to growth inhibition, morphogenetic abnormalities, and even death. However, different plant species show various radiosensitivity, which is also dependent on their ploidy. Moreover, there is evidence of a stimulating effect of low doses of X-rays or other types of ionising radiation on subsequent plant growth (Bayonove *et al*. 1984, De Micco *et al*. 2014a).

Macromolecules, in particular DNA, are critical targets of ionising radiation (Al-Enezi and Al-Khayri 2012). The DNA may be directly impaired or indirectly damaged due to the overproduction of free radicals, leading to DNA single- and double-strand breaks (Yokota *et al.* 2007). The latter are the main source of chromosomal aberrations, which determine changes in gene expression, followed by the alteration of plant structure and function (Kovalchuk *et al.* 2004). Actively growing tissues, and consequently organ structure, are very sensitive to radiation, since cell proliferation can be altered due to defects in mitosis (De Micco *et al.* 2011). At functional level, high doses of radiation severely affect photosynthetic processes, acting on all components of the photosynthetic apparatus, namely the light-harvesting complex, electron transport carriers and Calvin cycle enzymes (Kovacs and Keresztes 2002, Kim *et al.* 2004, Arena *et al*. 2014a).

In this study, we irradiated adult plants of dwarf tomato during blossoming with different doses of X-rays with the aim to investigate phenotypic responses in terms of leaf morpho-anatomical traits and photosynthetic efficiency in two types of leaves characterised by different lamina expansion at the time of irradiation. We hypothesise that leaves irradiated at different developmental stages respond with a different sensitivity to X-rays because of the different incidence of proliferating and enlarging cells in the lamina.

Moreover, as plants exposed to radiation at sub lethal doses may experience DNA alterations despite still showing a normal phenotype, we also conducted a genetic analysis by random amplified polymorphic DNA (RAPD)-PCR to evaluate if X-rays are able to trigger mutagenesis in actively-expanding leaf tissue (Dhakshanamoorthy *et al*. 2011, El-Sherif *et al.* 2011, Abdel Haliem *et al.* 2013). Indeed, it has been demonstrated that the RAPD-PCR, after proper optimisation, has a potential to detect various types of DNA damages and mutations, even those that do not prime phenotypical changes (Atienzar and Jha 2006). Different RAPD-PCR profiles are proxies of genetic differences among samples since different profiles are associated to different base composition in some points of the sample DNA.

Our specific aims were to assess: *1*) a threshold dose of X-rays at which morpho-anatomical changes, photosystem (PS) 2 impairment, and DNA alterations may be expected; *2*) whether different responses occur in leaves irradiated at different developmental stages; and *3*) possible relationships among radiation-induced DNA alterations, leaf anatomy, and PS 2 functionality.

Materials and methods

Plants, growth conditions, irradiation treatments, and sampling: The experiment was conducted on adult plants (at the blossoming stage) of dwarf tomato (*Solanum lycopersicum* L. cv. Microtom) grown from seeds (*Holland Online Vof*, Amsterdam, The Netherlands) in 10 cm diameter pots with peat and soil (1/1, v/v), in a greenhouse with mean day/night temperatures of 28.4/21.6 °C, mean photosynthetic photon flux density of 446 μmol (photons) m^{-2} s^{-1} recorded at noon at the canopy level, a 12-h photoperiod, and an air humidity of 55 %. The plants were irrigated at a 2-d interval to reintegrate the water lost by evapotranspiration, by reaching the container capacity. Details of the cultivation conditions and procedures are reported in De Micco *et al.* (2014b).

At the beginning of flowering (when one flower was blossoming on the plant), the plants were irradiated with different doses of X-rays (0.3, 10, 20, 50, and 100 Gy) at a dose rate of 1 Gy min^{-1} using a Thomson tube (*TR 300F*, 250 kVp, *Stabilipan*, *Siemens*, Forchheim, Germany), where X-rays were filtered by 1 mm-thick copper foil. Before irradiation, physical dosimetry was performed by using a ionisation chamber (*Victoreen*, Mödling, Austria). The distance between each plant and the X-ray generator was set up to have the established doses on two targets (leaf types) per each plant: *1*) adult leaves (L1, which showed a lamina size larger than 70 % of the theoretical lamina expansion, TLA) and actively developing leaves (L2, which showed a lamina size of about 25 % of the TLA). The TLA was established as the mean lamina expansion of fully developed leaves of the not-irradiated control plants measured in a preliminary cultivation trial. To evaluate the TLA, 3 single fully-expanded leaves from 5 control plants (for a total of 15 leaves) were photographed with a digital camera (*Nikon D3100*, *Nikon Europe*) and the area of leaf lamina was measured with the software program *AnalySIS 3.2* (*Olympus*, Hamburg, Germany). Leaves were considered fully expanded when two consecutive measurements (at 7-d interval) gave the same value of the leaf lamina surface. At the time of irradiation, L1 and L2 leaves corresponded to the fourth and fifth leaves inserted on the stem from the base. The area of leaf lamina (LA, leaf area) in L1 and L2 leaves from the irradiated and not-irradiated plants was measured, as reported above, when they reached the full expansion to build dose-response curves of the area of leaf lamina. After each irradiation treatment, in the case of L1 leaves, chlorophyll *a* fluorescence was immediately analysed, while sampling

for anatomical analyses was performed at the full lamina expansion. Regarding L2 leaves, the same ecophysiological analyses and RAPD profile determination were performed together with sampling for anatomical analyses two weeks later, at the full lamina expansion.

Microscopy and digital image analysis: One median leaflet was sampled from L1 and L2 leaves from five plants per treatment. The samples were fixed in 40 % (v/v) formaldehyde/glacial acetic acid/50 % (v/v) ethanol; 5/5/90 by volume). The subsamples of lamina (5 × 5 mm) were then dissected under a microscope (*SZX9*, *Olympus*), dehydrated in ethanol series (up to 95 %, v/v, ethanol) and embedded in acrylic resin *JB4* (*Polysciences*, Warrington, PA, USA). Cross sections (5 µm thick) were cut through a rotary microtome, stained with 0.025 % (m/v) Toluidine blue in a 0.1 M citrate buffer at pH 4 (Reale *et al.* 2012) and mounted with *Canadian Balsam*. The sections were analysed under a light microscope with transmitted light (*BX61*, *Olympus*). Unstained sections were mounted in mineral oil for fluorescence and observed under a UV-microscope (*BX60*, *Olympus*) equipped with a mercury lamp, a band-pass filter of 330 - 385 nm, a dichromatic mirror of 400 nm and above, and a barrier filter of 420 nm and above. Such settings highlight the presence of yellow-orange autofluorescent simple phenolics (Fukuzawa 1992, Ruzin 1999).

Images were collected by means of a digital camera (*XM50*, *Olympus*) and analysed by means of the *AnalySIS 3.2* (*Olympus*) software program to quantify the following anatomical traits: *1*) the thickness of palisade and spongy parenchyma (measured in 5 regions along the leaf lamina per section); 2) the cell area and shape of upper and lower epidermis, palisade, and spongy parenchyma (measured in 20 cells per each tissue per section). In detail, cell shape was characterised as an aspect ratio (elongation of a particle), and convexity (turgidity of a particle assuming a maximum value of 1) (De Micco *et al.* 2008, Van Buggenhout *et al.* 2008). A cell area occupied by simple phenolics was measured in four regions (of about 200 × 200 µm each) of mesophyll per section, avoiding veins. A surface occupied by such phenolic compounds was calculated as the percentage of tissue/picture occupied by yellow-orange autofluorescent compounds in UV-microscopy (De Micco and Aronne 2008).

Chlorophyll fluorescence and pigment content analysis: Fluorescence measurements (the quantum yield of PS 2 electron transport, Φ_{PS2}; the maximum efficiency of PS 2 photochemistry, F_v/F_m) and determination of photosynthetic pigment content were carried out on five fully expanded L1 and L2 leaves from the irradiated and control plants. The Φ_{PS2} and F_v/F_m were used as indicators of PS 2 functionality. Chlorophyll *a* fluorescence was measured using a pulse-amplitude-modulated fluorometer (*Junior-PAM*, Walz, Effeltrich, Germany), supplied with a monitoring leaf-clip *Junior-B* (*Walz*), after 30 min dark adaptation. Ground fluorescence, F_0, was induced on dark adapted leaves by blue LED internal light of about 2 - 3 µmol m^{-2} s^{-1} at a frequency of 0.5 kHz. The maximal fluorescence in dark-adapted leaves, F_m, was induced by a 1 s saturating light pulse (10 000 µmol m^{-2} s^{-1}) at a frequency of 10 kHz; the ratio of variable to maximal chlorophyll fluorescence (F_v/F_m) was calculated as $(F_m-F_0)/F_m$. For the fluorescence measurements in light-adapted leaves, each leaf was exposed to a photosynthetic photon flux density of 420 µmol m^{-2} s^{-1} for 5 min. Steady-state fluorescence, F_t, and maximal fluorescence, F_m', were measured, setting the internal fluorometer light at a frequency of 10 kHz. F_m' was determined by a 1 s saturating light pulse (8000 µmol m^{-2} s^{-1}). The quantum yield of PS 2 electron transport (Φ_{PS2}) was expressed according to Genty *et al.* (1989).

Total chlorophylls were extracted in ice-cold 100 % acetone. The extracts were centrifuged at 3 000 *g* in a *Labofuge GL* (*Heraeus Sepatech*, Hanau, Germany) for 5 min, and absorbance of the supernatants determined with a spectrophotometer (*UV-VIS Cary 100*, *Agilent Technologies*, Palo Alto, CA, USA). The content of pigments was calculated according to Lichtenthaler (1987).

Genomic DNA extraction and RAPD-PCR analysis: A RAPD-PCR analysis was performed on DNA extracted from L2 leaves, maintaining the extraction products separated for control and each irradiation treatment. The DNA was extracted using the procedure provided by a *DNeasy* plant mini kit (*Qiagen*, Hilden, Germany); DNA concentration and purity were determined by using a *NanoDrop* spectrophotometer (*Thermo Scientific*, Wilmington, USA). Polymerase chain reactions were performed in reaction mixtures of 25 mm^3 containing 5 ng of genomic DNA, 4 mm^3 of 5 µM primer, 200 µM dNTPs, 1 U Taq DNA polymerase (*Fermentas*, Waltham, MA, USA), and an appropriate amplification buffer. Negative control samples without the template DNA were always included to check for contamination.

The RAPD protocol, performed in a *MyCycler* thermocycler (*Bio-Rad*, Hercules, USA), consisted of an initial denaturing step at 94 °C for 4 min followed by 40 cycles at 94 °C for 45 s, 36 °C for 45 s, and 72 °C for 60 s, with an additional extension at 72 °C for 8 min. The PCR amplification products were separated on a 1.5 % (m/v) agarose gel in TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM Na_2EDTA). One kilobase DNA ladder (*Fermentas*) was used as a DNA molecular mass standard. The RAPD products were stained with ethidium bromide and photographed under UV-radiation by using the *Gel Doc ™XR* system (*Bio-Rad*).

Each RAPD product was separately screened for changes with 12 decamer primers (Table 1 Suppl.); in order to obtain significant and reproducible amplifications, all PCR reactions were repeated twice and independently. Furthermore, only strong, reproducible, and clearly distinguished bands were considered. A binary character matrix was built for each RAPD product,

with each band representing a character having a value of 1 (if the band was present in that lane) or 0 (absence of the band), following a conservative criterion for character selection (Belaj *et al*. 2004). The common approach in population genetics studies using the RAPD-PCR is using PCR products to produce a distance matrix in order to evaluate the genetic similarity of samples. Usually, the matrix is built using Nei's standard distance formulation (Nei 1978) based on a specific assumption on mutation and genetic drifts. Since the changes analysed here were not associated with population genetics dynamics, a simpler and more generic Euclidean metrics was applied. Thus, different character matrices were joined and used as an input for calculation of an Euclidean distance matrix.

To visualise the distance among samples, the matrix was subsequently expressed as an unweighted pair group method with arithmetic mean dendrogram. The two aforementioned data analysis techniques were carried out in the R statistical environment: the distance matrix was calculated using the built-in dist function, whereas the unweighted pair group method with arithmetic mean endrogram was performed with the hclust function and displayed using *plot.phylo* from the *ape* package, *v. 3.3* (Paradis *et al*. 2004).

Statistical analysis: The anatomical and physiological data were firstly processed with a two-way *ANOVA* using a dose of X-rays (D) and leaf type (L) as independent factors, also showing their interactions (D × L). Then, to highlight the dose-response trends in leaves irradiated at the two different developmental stages (L1 and L2), data were *a-priori* separated according to the leaf type and processed with one-way *ANOVA* using an X-ray dose as a factor. The Student-Newman-Keuls coefficient was applied for multiple comparison tests (α = 0.05). The Kolmogorov-Smirnov and Shapiro-Wilk tests were performed to check for normality. The percent data were transformed through an arcsine function before statistical analysis. The *SPSS*® statistical package was used (*SPSS Inc.*, Chicago, IL, USA) for all analyses.

Results

Leaf area was not significantly affected by the interaction between the X-ray dose and leaf type (D × L), whereas the main effects of both D and L on lamina expansion were significant (Table 2 Suppl.). L1 leaves almost completed their lamina expansion at all the doses of X-rays, reaching up to 95 % of the TLA at 100 Gy. On the contrary, the X-rays reduced development of L2 leaves, which expanded only up to 75 % of TLA at 100 Gy. The minimum lamina expansion was observed in L2 leaves irradiated at 20 Gy (Fig. 1). A relation between the leaf area and X-ray dose was not linear for both leaf types.

The plants irradiated at 50 and 100 Gy underwent an early death and did not complete the life cycle: indeed, shoots, leaves and blossoming flowers underwent necrosis indicated by an early change in colour and desiccation. Qualitative anatomical traits of both L1 and L2 leaves were not affected by the irradiation (Fig. 2). Indeed, all analysed leaves were characterised by a typical dorsiventral structure where the whole tissue did not show any signs of structural damage. The X-rays did not hamper tissue differentiation also in leaves which were actively developing at the time of the irradiation (Fig. 2*G-N*). Quantification of anatomical features shows that the irradiation caused certain modifications not always linearly dependent on a dose. Lamina thickness and cell area of the whole tissue but lower epidermis were significantly affected by the D × L interaction; the main effects of both D and L were also significant (Table 2 Suppl.). The cell shape of palisade parenchyma was significantly affected by the D × L interaction (Table 2 Suppl.)

In L1, the doses of 0.3 and 10 Gy were responsible for a significantly thinner lamina than in the control (Fig. 3). Similar X-ray-induced trends were observed in the cell size of upper epidermis (Fig. 4*A*). The cell sizes of lower epidermis and spongy parenchyma were significantly enlarged only at the dose of 100 Gy (Fig. 4*B*, *D*), whereas the cells of palisade parenchyma were significantly increased by the 50 and 100 Gy doses (Fig. 4*C*). The cells of upper epidermis and palisade parenchyma tended to be more elongated (higher values of the aspect ratio) after irradiation at 50 and 100 Gy compared to the control (Table 1). No significant differences were found in cell elongation in the other analysed tissues.

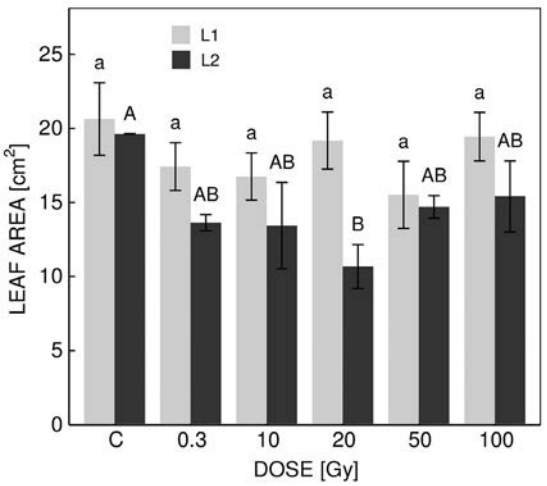

Fig. 1. The leaf area of nearly mature (L1) and developing (L2) leaves from control and X-ray irradiated plants of *Solanum lycopersicum* L. cv. Microtom. Means ± SEs, n = 15. Different letters indicate significantly different values according to multiple comparison tests ($P < 0.05$). Small and capital letters refer to L1 and L2, respectively.

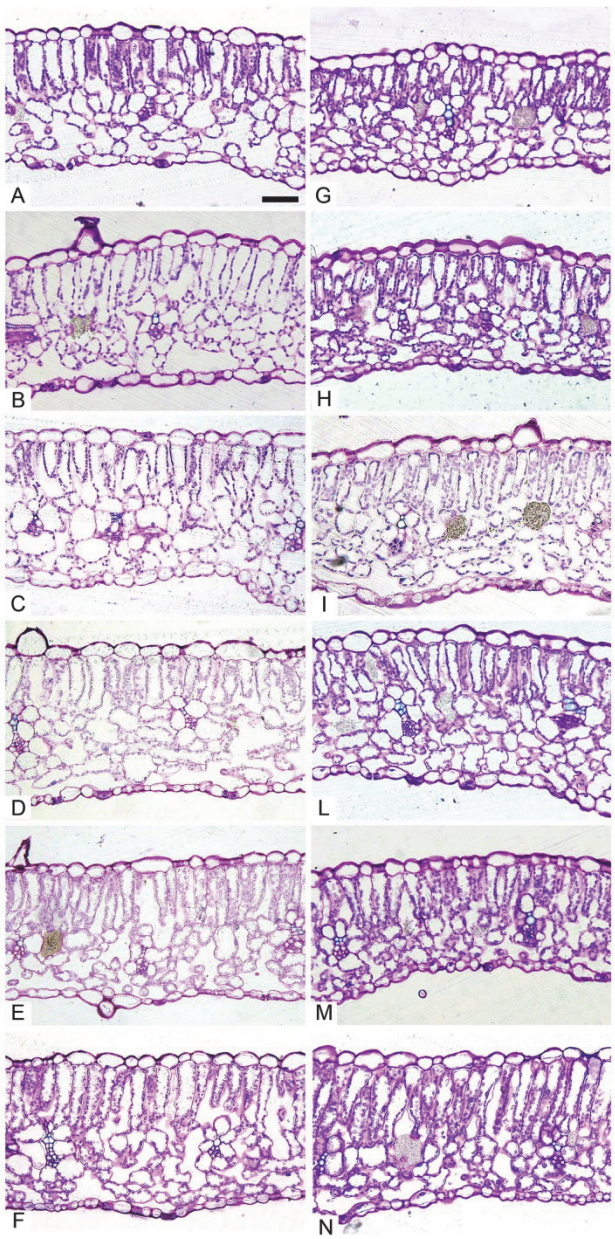

Fig. 2. Light microscopy views of cross sections of nearly mature (*A-F*) and developing (*G-N*) leaves of *Solanum lycopersicum* from control (*A*, *G*) and irradiated leaves (*B-F*, *H-N*) at different X-ray doses: 0.3 Gy (*B*, *H*), 10 Gy (*C*, *I*), 20 Gy (*D*, *L*), 50 Gy (*E*, *M*), and 100 Gy (*F*, *N*). All images are at the same magnification. *Bar* = 100 μm.

In L2 leaves, a dose-dependent trend in increasing lamina thickness was found, leading to significantly thicker leaves in the plants irradiated with 50 and 100 Gy (Fig. 3). The increase in lamina thickness at the high doses of irradiation was accompanied by increases, not always significant, in cell sizes of palisade and spongy tissues (Fig. 4*C*,*D*). The cell sizes of upper and lower epidermis were either not affected or decreased after the irradiation (Fig. 4*A*,*B*). The cell aspect ratio remained almost unchanged at all the doses in all the tissues but palisade parenchyma (Table 1). As regards cell convexity, although significantly lower values were found at the different doses in the various tissues in both L1 and L2, measured values were always high (close to 1), thus indicating a high cell turgidity (Table 1).

The distribution of phenolic compounds, localised along cell membranes, was not influenced by the irradiation in both the leaf types. In quantitative terms, the percentage of tissue occupied by the autofluorescent phenolics was not significantly affected by the D × L interaction, but the main effects of both factors were highly significant (Table 2 Suppl.). In both the leaf types, the percent of phenolic compounds increased after the irradiation irrespective of X-ray dose (Fig. 5).

The analysis of the effect of different doses of X-rays on Φ_{PS2} and F_v/F_m in both L1 and L2 leaves (Fig. 6*A*,*B*) shows that PS 2 functionality was not differently affected in the two phases of leaf lamina expansion. More specifically, these parameters were not affected by the D × L interaction, whereas the main effect of the X-ray doses was significant (Table 2 Suppl.). Both L1 and L2 leaves exhibited a significant decreases in Φ_{PS2} and F_v/F_m after irradiation at 50 and 100 Gy if compared to the not-irradiated controls (Fig. 6*A*,*B*).

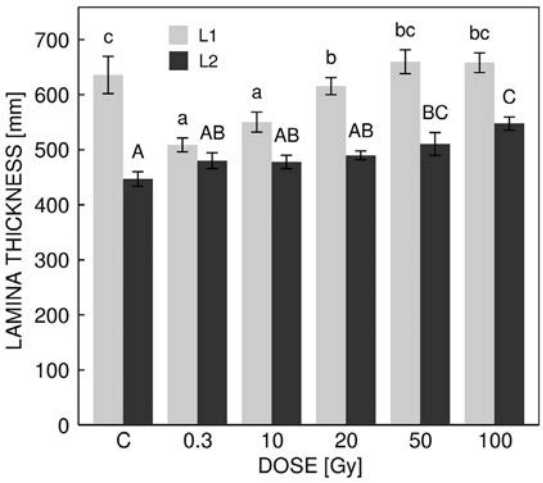

Fig. 3. The lamina thickness of nearly mature (L1) and developing (L2) leaves from control and X-ray irradiated plants of *Solanum lycopersicum* cv. Microtom. Means ± SEs, $n = 25$. Different letters indicate significantly different values according to multiple comparison tests ($P < 0.05$). Small and capital letters refer to L1 and L2, respectively.

Total chlorophyll content was neither affected by the D × L interaction nor by the leaf type as the main effect (Table 2 Suppl.). In L1 leaves, chlorophyll (*a*+*b*) content was not affected by the different doses of X-rays. In L2 leaves, the different doses showed different effects: the lowest chlorophyll content was found in samples irradiated at 50 and 100 Gy, whereas the maximum values were measured in leaves exposed to 10 and 20 Gy (Fig. 6*C*).

All the primers amplified successfully the genomic DNA from all tested samples (Fig. 7). Four out of

12 used primers Operon Primer B (namely; OPB-1, 4, 8, and 11) showed clear variations of the banding patterns with respect to the control sample, with 36 % of bands being polymorphic. The distance matrix (Table 2) and the dendrogram (Fig. 1 Suppl.) based on the RAPD-PCR data show that the 0.3 Gy dose was considered equal to 10 Gy (d = 0), and both are close to the control (d = 0.182). Moreover, 50 Gy was similar to 100 Gy, and both are separated from the other doses. Finally, the 20 Gy dose was classified as similar (d = 0.125) to the higher doses (50 and 100 Gy).

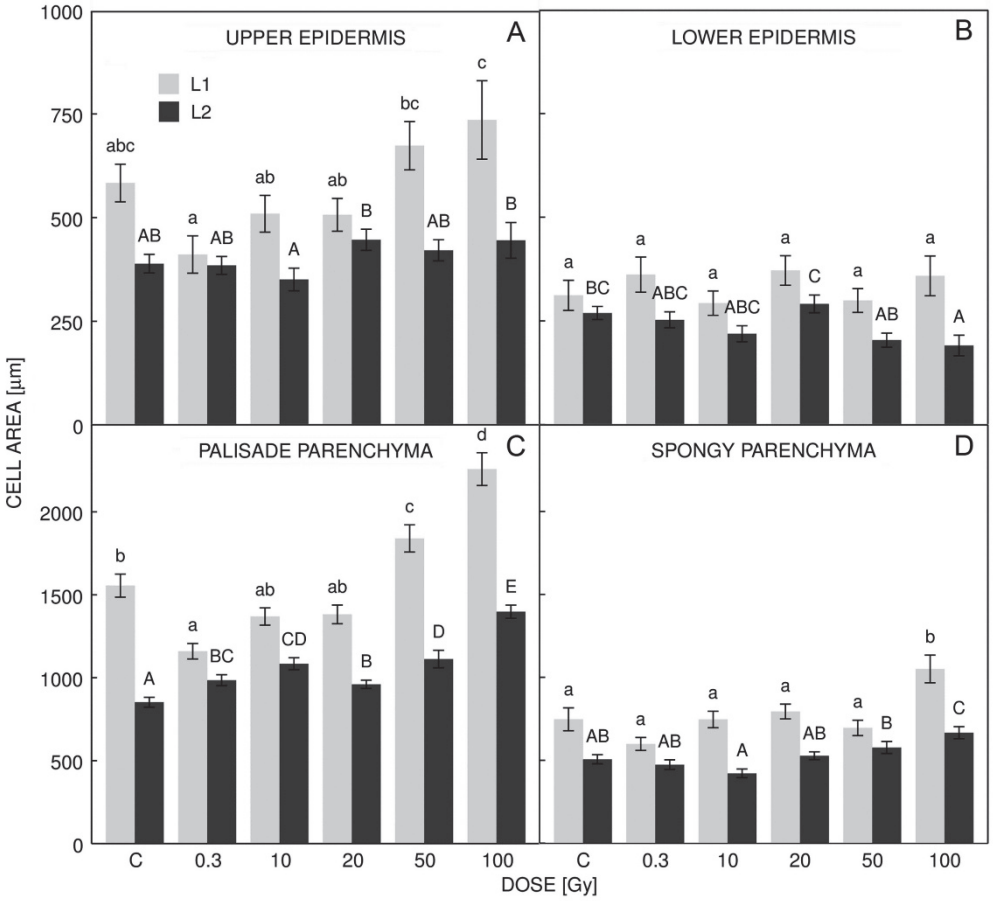

Fig. 4. The cell area of upper epidermis (*A*), lower epidermis (*B*), palisade parenchyma (*C*), and spongy parenchyma (*D*) of nearly mature (L1) and developing (L2) leaves from control and X-ray irradiated plants of *Solanum lycopersicum* cv. Microtom. Means ± SEs, $n = 100$. Different letters indicate significantly different values according to multiple comparison tests ($P < 0.05$). Small and capital letters refer to L1 and L2, respectively.

Discussion

Morphological and physiological alterations induced by ionising radiation can become evident when plants are exposed to doses of radiation exceeding the threshold dose for resistance, which is dependent on species and target organ (Holst and Nagel 2004, De Micco *et al*. 2011). In *S. lycopersicum* cv. Microtom, mature leaves (*i.e*., exposed to X-rays when the lamina was close to full expansion - L1) and young leaves (*i.e*., still actively developing at the time of irradiation - L2) showed different sensitivity to X-rays. An inhibition of leaf lamina expansion was induced by the irradiation in L2 leaves. Some modifications in quantitative anatomical traits were induced by the X-rays, especially regarding an increasing mesophyll thickness and cell size of palisade and spongy tissues. The different responses of L1 and L2 leaves to the irradiation may be considered as a consequence of a different number of cells in the proliferation and enlargement phases occurring in the two leaf types (Donnelly *et al*. 1999). Indeed, development of leaf lamina is a complex process where cell division and enlargement occur with specific spatial and temporal patterns mediated by transcription factors and hormones (Ben-Gera *et al*. 2016). Mitotic activity (either in periclinal or anticlinal cell division) in the layered tissue organisation can continue until the lamina reaches a half to three quarters of its final size (Steeves and Sussex

1989). It is likely that development of L1 leaves after the X-ray exposure mainly relied upon cell enlargement or on seldom cell proliferation, whereas expansion of the L2 leaf lamina was still dependent on mitotic activity of the plate, marginal and intercalary meristems (Esau 1965, Ichihashi and Tsukaya 2015), which are more prone to undergo genetic alterations than not-dividing cells.

Lack of precise dose-dependent tendencies of variation and the different responses found in the different tomato leaf tissues are in agreement with previous studies where quantitative anatomical traits of leaves were analysed in plants irradiated with X-rays on different target organs (De Micco *et al*. 2014a,b). The general trend of increasing lamina thickness and cell size in the certain tissues of the tomato leaves exposed at the high doses of radiation is in agreement with data reported in leaves of dwarf bean directly subjected to X-rays (De Micco *et al.* 2014a). Moreover, the high cell turgidity observed in all the tissues supports the idea that cell enlargement could be less constrained in irradiated leaves. This effect was probably due to a reduced cell wall resistance, which is a common phenomenon in tissue subjected to ionising radiation (Kovács and Keresztes 2002). The remarkable increase in autofluorescent phenolic compounds in mesophyll, even at low doses, is in agreement with the common strategy of enhancing production of such compounds acting as natural screens to protect tissues against a high radiation (Levine *et al.*

Table 1. Cell shape in different tissues of nearly mature (L1) and developing (L2) leaves of *Solanum lycopersicum* irradiated with different doses of X-rays. Means ± SEs, n = 100. Different letters indicate significantly different values according to multiple comparison tests ($P < 0.05$).

	X-rays [Gy]	L1 aspect ratio	convexity	L2 aspect ratio	convexity
Upper epidermis	0	1.615 ± 0.046 [a]	0.952 ± 0.008 [b]	1.625 ± 0.073 [a]	0.950 ± 0.007 [a]
	0.3	1.602 ± 0.070 [a]	0.956 ± 0.012 [b]	1.767 ± 0.061 [a]	0.946 ± 0.009 [a]
	10	1.777 ± 0.061 [ab]	0.955 ± 0.008 [b]	1.613 ± 0.056 [a]	0.947 ± 0.007 [a]
	20	1.669 ± 0.061 [ab]	0.932 ± 0.011 [a]	1.725 ± 0.056 [a]	0.948 ± 0.008 [a]
	50	1.832 ± 0.073 [b]	0.955 ± 0.009 [b]	1.847 ± 0.087 [a]	0.936 ± 0.011 [a]
	100	1.828 ± 0.090 [b]	0.956 ± 0.010 [b]	1.672 ± 0.064 [a]	0.954 ± 0.009 [a]
Palisade parenchyma	0	3.201 ± 0.081 [a]	0.863 ± 0.010 [b]	3.572 ± 0.080 [a]	0.835 ± 0.012 [a]
	0.3	3.572 ± 0.125 [ab]	0.854 ± 0.011 [ab]	3.624 ± 0.097 [a]	0.841 ± 0.011 [a]
	10	3.564 ± 0.077 [ab]	0.857 ± 0.010 [ab]	3.759 ± 0.082 [ab]	0.857 ± 0.009 [a]
	20	3.442 ± 0.082 [a]	0.830 ± 0.009 [a]	3.977 ± 0.077 [b]	0.857 ± 0.007 [a]
	50	3.930 ± 0.108 [b]	0.860 ± 0.010 [ab]	3.519 ± 0.127 [a]	0.854 ± 0.012 [a]
	100	3.877 ± 0.133 [b]	0.846 ± 0.013 [ab]	4.525 ± 0.118 [c]	0.840 ± 0.011 [a]
Spongy parenchyma	0	1.705 ± 0.065 [a]	0.922 ± 0.010 [ab]	1.724 ± 0.060 [a]	0.911 ± 0.010 [a]
	0.3	1.668 ± 0.064 [a]	0.912 ± 0.011 [a]	1.644 ± 0.043 [a]	0.925 ± 0.008 [ab]
	10	1.726 ± 0.067 [a]	0.929 ± 0.009 [ab]	1.558 ± 0.050 [a]	0.937 ± 0.008 [b]
	20	1.587 ± 0.049 [a]	0.925 ± 0.008 [ab]	1.664 ± 0.043 [a]	0.936 ± 0.008 [b]
	50	1.610 ± 0.057 [a]	0.942 ± 0.009 [b]	1.674 ± 0.073 [a]	0.936 ± 0.009 [b]
	100	1.626 ± 0.076 [a]	0.928 ± 0.010 [ab]	1.661 ± 0.075 [a]	0.926 ± 0.011 [ab]
Lower epidermis	0	1.517 ± 0.062 [a]	0.947 ± 0.011 [a]	1.512 ± 0.057 [a]	0.950 ± 0.010 [a]
	0.3	1.782 ± 0.095 [a]	0.952 ± 0.010 [a]	1.743 ± 0.080 [a]	0.945 ± 0.009 [a]
	10	1.625 ± 0.090 [a]	0.965 ± 0.008 [a]	1.729 ± 0.078 [a]	0.949 ± 0.008 [a]
	20	1.659 ± 0.062 [a]	0.955 ± 0.009 [a]	1.495 ± 0.051 [a]	0.950 ± 0.011 [a]
	50	1.625 ± 0.060 [a]	0.956 ± 0.010 [a]	1.752 ± 0.078 [a]	0.944 ± 0.010 [a]
	100	1.727 ± 0.091 [a]	0.952 ± 0.010 [a]	1.601 ± 0.059 [a]	0.952 ± 0.012 [a]

Table 2. Euclidean distance matrix for *Solanum lycopersicum* irradiated samples. Row and column labels are the doses of X-rays.

Dose [Gy]	0	0.3	10	20	50
0.3	0.1818182				
10	0.1818182	0.0000000			
20	0.2727273	0.3000000	0.3000000		
50	0.3636364	0.2222222	0.2222222	0.1250000	
100	0.3636364	0.2222222	0.2222222	0.1250000	0.0000000

2001, Lattanzio *et al.* 2008). An increase in phenolics has been also reported in leaves of dwarf bean, but only at very high doses of X-rays (De Micco *et al.* 2014a). Although not truly linearly dependent on dose, the modification of quantitative anatomical traits in the tomato leaves was often more severe at the high doses of radiation and was accompanied by the significant decrease in total chlorophyll content and PS 2 functionality especially in L2 leaves. These results are consistent with data reported for *Phaseolus vulgaris* leaves in which exposure to high doses of X-rays causes a strong reduction in photochemistry and in Rubisco activity in young but not in mature leaves (Arena *et al.* 2014a).

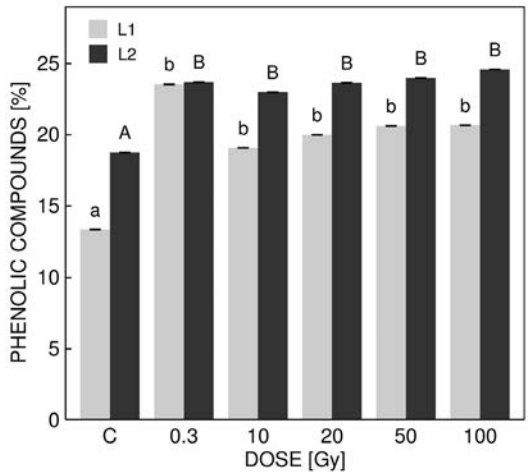

Fig. 5. The percentage of mesophyll occupied by phenolic compounds in nearly mature (L1) and developing (L2) leaves from control and X-ray irradiated plants of *Solanum lycopersicum* cv. Microtom. Means ± SEs, n = 20. Different letters indicate significantly different values according to multiple comparison tests ($P < 0.05$). Small and capital letters refer to L1 and L2, respectively.

Considering that young leaves are less resistant than mature leaves to X-rays because their tissues have generally more cells in active proliferation and they may be damaged by irradiation (Steeves and Sussex 1989), we analysed L2 leaves by RAPD-PCR to detect possible DNA alterations. Although more than a half of the primers chosen for this study did not evidence any changes following the tissue exposure to X-rays, the remaining primers confirmed that the effects of X-rays were more severe at the high doses of radiation similarly as found for anatomical and eco-physiological traits. The RAPD-PCR profiles evidenced the presence of polymorphic bands in all irradiated young leaves compared to the control, confirming the mutagenic effect exerted by X-rays on young leaf tissues (Danylchenko and Sorochinsky 2005). The distance matrix and the dendrogram reveal that doses of 0.3 and 10 Gy induced a low DNA alteration power, 20 Gy dose increased alteration power, which reached a plateau at 50 - 100 Gy. This suggests a sigmoid trend in the dose-effect (*i.e.*, the degree of genetic differences) relationship. The occurrence of very close 50 and 100 Gy clusters suggests similar outcomes, whereas 20 Gy placed on an independent cluster indicates a different genomic response. The genetic structure found in the population of the samples corroborate the anatomical and eco-physiological results: 20 Gy seems to mark the threshold dose above which genetic alterations, morpho-anatomical modifications, and perturbations in the photosynthetic apparatus became substantial, especially in actively developing leaves. The equivalence of higher doses (50 and 100 Gy) was evident at all analysed levels, namely morphological, eco-physiological, and genetic.

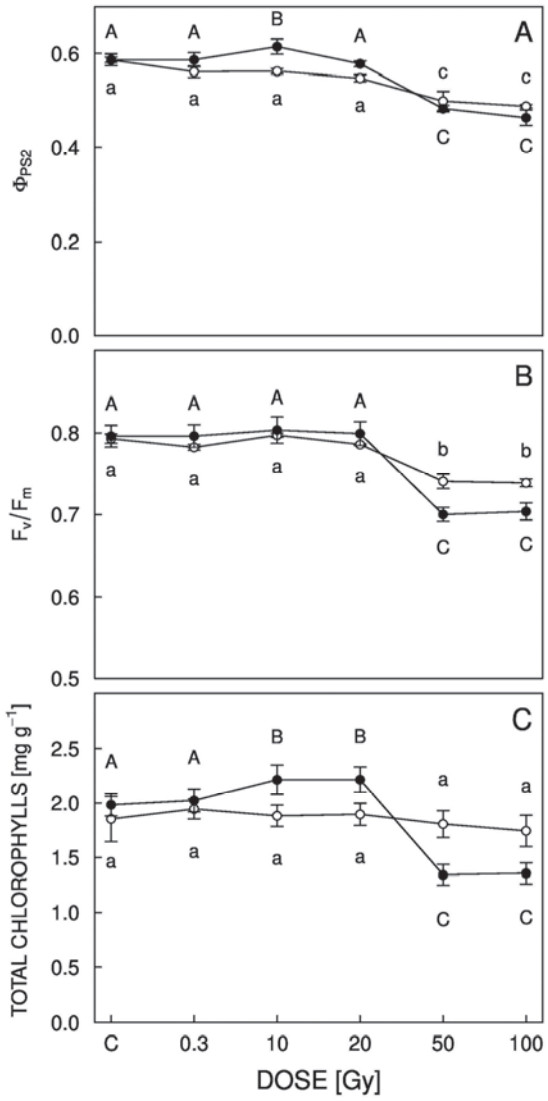

Fig. 6. Quantum yield of PS 2 linear electron transport, Φ_{PS2}, (*A*), maximum efficiency of PS 2 photochemistry, F_v/F_m, (*B*), and content of chlorophyll *a*+*b* (*C*) in nearly mature (L1 ○) and developing (L2 ●) leaves from control and X-ray irradiated plants of *Solanum lycopersicum* cv. Microtom. Means ± SEs, n = 5. Different letters indicate significantly different values according to multiple comparison tests ($P < 0.05$). Small and capital letters refer to L1 and L2, respectively.

In conclusion, the overall analysis shows that the modifications of the quantitative anatomical traits induced by the X-rays in tomato leaves were not truly linearly dependent on the dose, whereas the response of the photosynthetic apparatus, in terms of photochemistry and chlorophyll content, was more strictly dose-dependent. Among doses, 20 Gy may be considered as the threshold dose of X-rays at which phenotypic alterations become more evident. The DNA rearrangements in L2 leaves subjected to the high doses of X-rays could be responsible for the decline of photochemistry and leaf pigment content as well as for the reduced lamina expansion, confirming our hypothesis of a higher sensitivity of young leaves due to the presence of a high number of cells in active proliferation that may be damaged by irradiation.

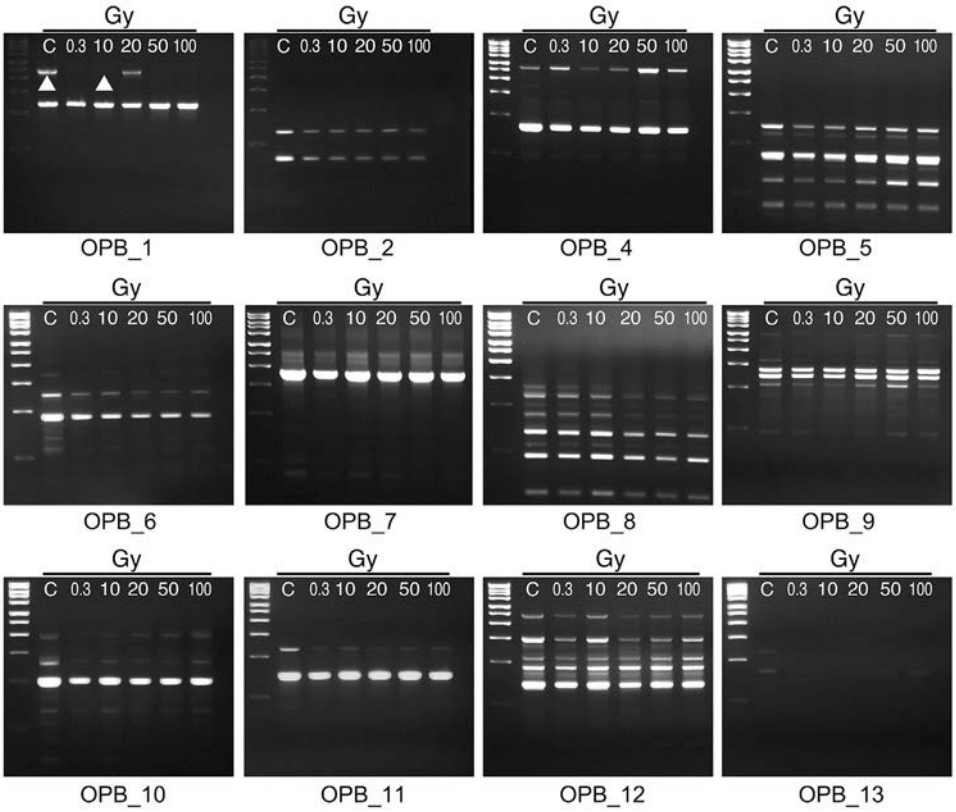

Fig. 7. Random amplification of polymorphic DNA (RAPD) profiles of genomic DNA in developing (L2) leaves (n = 5) from control and X-ray irradiated plants of *Solanum lycopersicum* cv. Microtom obtained by using specific Operon Primers type B (OPB) primers. In OPB-1, the *arrows* indicate the presence/absence of the bands as an example.

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