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Biofortified tomato plants as a test bed for space agriculture

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Summary

Future long-term space missions will focus to the solar system exploration with the Moon and Mars as the leading goals. As already planned by the Artemis program led by NASA and supported by the main Space agencies, Lunar and Martian missions will be characterized by extended duration. In future deep-space manned missions, when resources or supplies are limited, novel methods for production of edible biomass and recycling of resources are expected to provide solutions for long-term crew survival. During these missions, higher plants will take a crucial role as they produce oxygen, reduce carbon dioxide and recycle water, as well as being a fundamental fresh food source for a nutritionally balanced crew diet, actively contributing to the crew psychological well-being, as well.

Isolation, limited resources, microgravity and ionizing radiation (IR) are considered the main hazards of spaceflights due to their capacity to generate cell metabolic stress. IR are the most prominent pro-oxidant stimuli that plants have to cope with because they could both directly damage the DNA structure or generate free radicals through the radiolysis process. The issue of counteracting overproduction of free radicals generated by harmful IR is crucial not only for plant development but also for human survival in space outposts and, in the latter sense, opens the way to the ideal ‘antioxidant space fresh food’. Therefore, there is great interest in the development of biofortified plants accumulating natural antioxidants that may be introduced with the diet of astronauts, as well.

Among secondary metabolites that give protection against environmental challenges, anthocyanins are well-known for their potent anti-oxidant properties and, recently, they have been proposed as a component of antioxidant formulations to be administered during spaceflight to maintain space explorers’ health. Indeed, efforts to figure out how plants might perform predictably upon extraterrestrial environment and, possibly, to support life in the confined and unhealthy environment of a space outpost, are needed.

This PhD thesis pioneers the development of plant ideotypes aimed at space application through the design of tomato plants (cv. MicroTom) expressing the R2R3-MYB transcription factor *PhAN4* from *Petunia hybrida*. Tomato (*Solanum lycopersicum* L.) is one of the most cultivated vegetable worldwide and its fruits are the largest dietary source of several nutraceutical metabolites. In particular, the dwarf cultivar MicroTom is one of the crops that meets many of the specific requirements for space cultivation. Despite the richness in valuable compounds known for their nutraceutical effects, accumulation of flavonoids in tomato fruits is sub-optimal and, with some exception, anthocyanins are not present. To elucidate if the *PhAN4* gene was able

to restore the anthocyanin production, a simplified MicroTom-derived hairy root cultures (HRCs) model was established and then characterized using a transcriptome-wide RNA-seq approach and mass spectroscopy analysis. The constitutive expression of *PhAN4* in the HRCs model resulted in modulation of gene expression related the anthocyanin biosynthetic pathway together with modulation of gene expression related to abiotic, biotic and redox stimuli. In addition, AN4 HRCs showed significantly higher capability to counteract ROS accumulation and protein misfolding upon a high pro-oxidant stimulus (i.e., gamma rays) compared to controls. These results paved the way to whole plant engineering. Transgenic lines harboring a single copy of the transgene inserted respectively in the chromosome 8 and 12 were characterized for the *PhAN4* ectopic expression. Both homozygous AN4-M and AN4-P plants, and, in particular, the hemizygous AN4-P₂ line, showed a purple pigmentation throughout their structure, from leaves and leaf veins, to stem and roots, to anthers and ovary. The phenotypic characterization revealed that the engineered genotypes were unchanged in life cycle as compared to the untransformed control and retained the dwarf phenotype (even smaller) while maintaining the same yield. Total anthocyanin content evaluation and DPPH assay demonstrated the accumulation of anthocyanins in fruits and the increase in the antioxidant capacity compared to wild type. Engineered fruits were also evaluated by Electron Spin Resonance Spectroscopy (ESR) and Photoluminescence analysis. These analyses showed lower accumulation of reactive oxygen species and decreased protein folding damage compared to wild type, upon an *ex vivo* pro-oxidant stimulus (i.e., 2 kGy of gamma rays). To study how the engineered MicroTom could possibly perform upon ionizing radiation, we exposed seeds and plants at two different developmental stages (30 and 45 DAS) at doses that plants would possibly experience during a long-term space mission (0 Gy, 0.5 Gy, 5 Gy or 30 Gy of gamma rays originated from a ⁶⁰Co source).

These experiments allowed us to tackle the lack of knowledge regarding the effects of gamma rays on fully developed tomato plants and on their offspring.

Plants response to gamma rays were evaluated following two approaches: biometric parameters were monitored to detect changes occurred at both phenotypic and phenological level and multiparametric fluorescence-based indices were used to indirectly measure changes occurred at physiological level in a non-destructive manner.

Results showed that irradiation of the dry seeds did not hamper germination and seed-to-seed cycle, demonstrating that, at this phenological stage, seeds are more resistant to ionizing radiation due to their structure. Experiments conducted on plants irradiated at 30 and 45 DAS revealed that plants can tackle gamma rays doses until 5 Gy through adjustments at the physiological level. Indeed, low doses of ionizing radiation (0.5 and 5 Gy) delivered at both seed stage and

fully developed plants do not impair plants growth and, in some cases, can led to beneficial traits (e.g., smaller size or increased number of seeds). Conversely, between 5 and 30 Gy there is a limiting dose that compromise the plant development and the production of fruits and viable seeds.

Interestingly, fluorescence-based indices (i.e., SFR, NBI, FLAV and ANTH) allowed us to detect a bi-phasic response to IR in plants irradiated at 30 DAS and a less pronounced response in plants irradiated at 45 DAS. This 'lack of response' could be due to an age-related resistance to stress, highlighting the different effect of gamma rays delivered to plant at different developmental stages.

Irradiation of plants at 30 DAS also affected different parameters on offspring, with changes that may be ascribed to epigenetic modifications and altered frequency of transitional recombination. Lastly, gamma rays, independently from the genotype and developmental stage, did not caused alteration in the efficiency of photosynthetic apparatus.

In conclusion, among the three genotypes considered, AN4-M plants, maybe to the favourable genomic asset and the favourable content of anthocyanins, demonstrated a high capacity to overcome the stress of gamma rays exposure.

All together, the information obtained contribute to advance our understanding of space effects on plants, opening the way to the development of novel plant ideotypes with enhanced favourable traits suitable for future long-term space mission.

1. INTRODUCTION

1.1 The Space Colonization Era

On 12th April 1961, Yuri Gagarin travelled outer space becoming the first human to orbit the Earth. Since this event, the United States of America and the Soviet Union competed on either geopolitical and scientific level to reach the Moon, inaugurating the Space Colonization Era. Fifty years after the first human footprints on Moon, humanity returned its gaze to outer space with the new goal of establishing a semi-permanent human presence through lunar bases and orbiting stations. In the long-term, the US, Europe, China, Russia and India hope to use the Moon as a springboard for human travel to Mars. Thankfully, the willingness to invest money and manpower to the development of human spaceflight activities is not only limited to geopolitical interests and to inaugurate ‘niche’ endeavours such as space tourism (as happened in July 2021, thanks to the private companies Virgin Galactic and Blue Origin) (Figure 1.1), but is expected to foster economic development through the creation of jobs, the enhancement of research and the development of innovative technologies, as well.

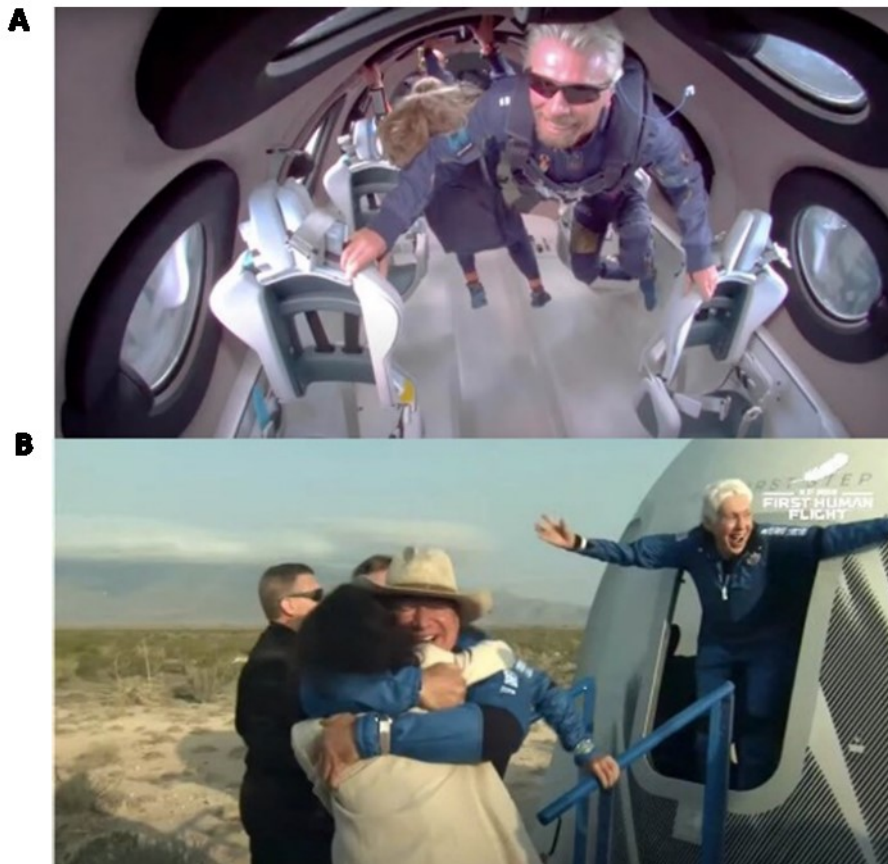


Figure 1. 1 (A) Virgin Galactic rocket plane after returning from reaching the edge of space (i.e., 80 km off the ground, towards the upper part of the mesosphere, as per US military, the Federal Aviation Administration and NASA definition). (B) The rocket ship New Shepard at the end of a short journey to space.

To all these ends and as a step in the path to Mars colonization, NASA, with the Artemis program, plans to build a station similar to the International Space Station (ISS, where, currently, astronauts are allowed to spend more than 6 months, in the outer space) around the Moon, the ‘Lunar Orbital Platform-Gateway’, to extend human presence in space and to provide a reliable platform for scientific experiments (Figure 1.2).

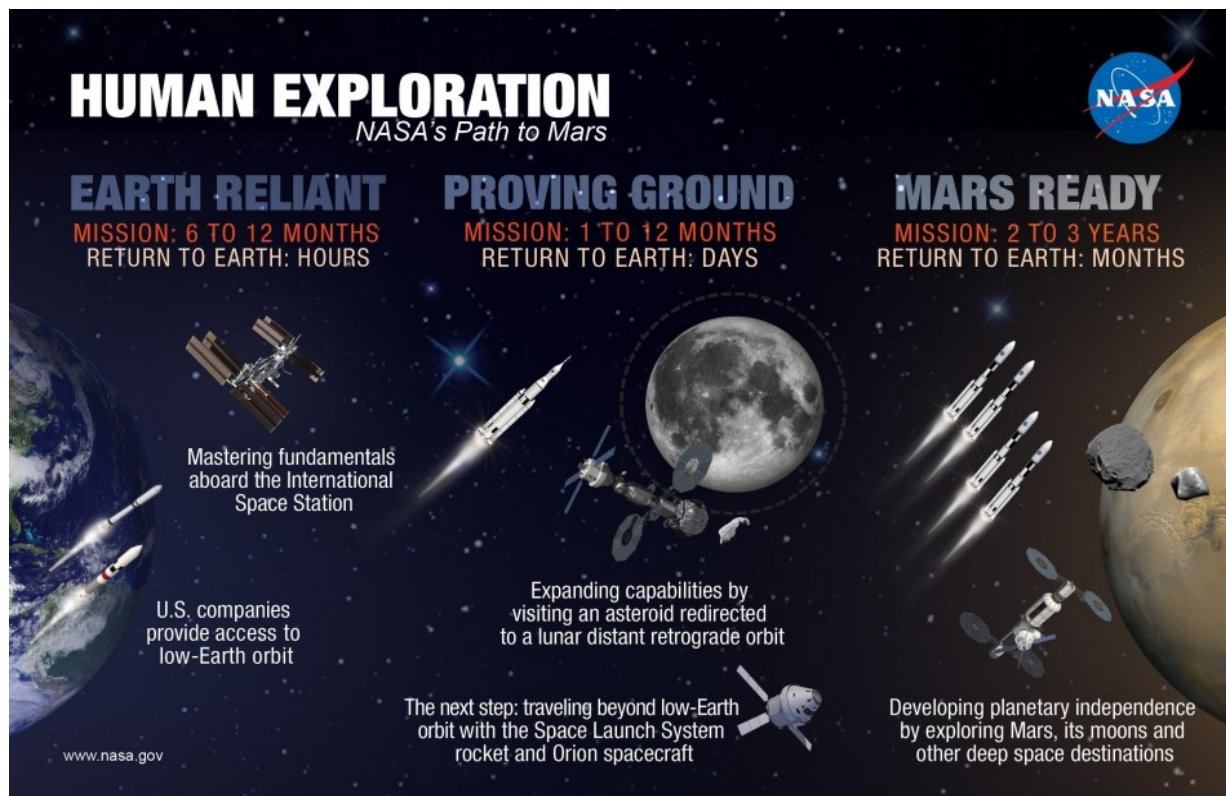


Figure 1. 2 NASA’s Path to Mars: a steady stepping stone approach to meet the challenge of advancing deep space technologies through the Asteroid Redirect Mission, ©NASA.

As space manned missions become longer and deeper into the solar system, frequent resupplies for life support will become difficult and onerous. To face this issue, Bioregenerative Life Support Systems (BLSSs), a habitation environment able to simulate the Earth’s biosphere, will be needed (De Pascale et al., 2021a).

1.1.1 How plants are expected to assist human life in space

As said, members of the human species will venture within the solar system, most likely focusing on Moon and Mars. Despite similarities with current space missions and analogues, lunar and Martian missions will be complicated by the extended durations and distances due to logistics and radiation concerns, among others. As man moves into outer space, plants would need to follow, to allow the setup of BLSSs able to recycle resources such as oxygen and water. Besides this, plants will be necessary to provide also healthy food and, possibly, medicines to the crew

(i.e. by exploiting plants as biofactories of value-added metabolites, antioxidants, vaccines and antibodies). BLSSs are artificial micro-environments divided into several compartments in which physical, chemical and biological processes are integrated to generate an Earth-like environment able to support human life in space (Zabel et al., 2016).

Studies on how to improve BLSSs would increase the knowledge on the regeneration of essential resources obtained from biological processes on Earth, as well (Monje et al., 2003). Indeed, the bioconversion of waste into a soil-like substrate, the nitrogen recovery from urine and higher plants cultivation, among others, represent the most important goals to reach advances both in space unit technologies and on Earth (Fu et al., 2016). Recycling processes will have to include food production coupled with oxygen (O₂) and water (H₂O) recovery systems that will imply the use of one dedicated compartment for producing edible biomass (Gitelson, 1992). Within BLSSs, higher plants will have an essential role in oxygen production, carbon dioxide uptake and water purification (Figure 1.3) (Arena et al., 2021; De Pascale et al., 2021b). Very importantly, higher plant cultivation will recycle nutrients derived from human wastes and will provide fresh food and a possible source of pharmaceutical value molecules, as a healthy supplement to astronauts' diet and medicines supplies, that, otherwise, would be only strictly dependent on the initially available payloads. Finally, plants are also expected to provide psychological support for crewmembers forced to live in a confined environment (Paradiso et al., 2014).

1.1.2 The choice of plant candidates to sustain human life in space

Successful growth, development and reproduction of higher plants in space rely on appropriate availability and uptake of water and nutrients, and efficient energy distribution through the photosynthesis process and gas exchange (Wolff et al., 2013). The perfect candidates are crop varieties that meet some specific requirements, as recently summarized by Dueck et al., 2016 and Arena et al., 2021.

performances of plants in space conditions is pivotal to develop the appropriate technologies and cultural conditions for providing the optimal plant yield (Monje et al., 2003).

Since the beginning of manned space flight, a wide range of experiments on higher plant performances have been conducted, together with ground-based trials, on board the space shuttle, on biosatellites and orbital stations. The design of growth facilities and the history of plant cultivation in space was recently reviewed in Zabel et al., 2016 (Figure 1.4).

The first plant growth system was the Oasis 1 on board the Soviet Salyut station in 1971. Oasis 1 was designed with eight cultivation slots containing chinese cabbage, leek, onion and flax that were illuminated by fluorescent lamps. In July 1975, onions were grown on the Oasis 1M (Figure 1.5, left), an upgraded version of the Oasis 1, resulting in the first space-grown vegetables ever eaten by humans (Harvey and Zakutnyaya, 2011). The first experiment specifically designed to investigate the psychological benefits of plants cultivation on crewmembers was the Malachite growth system, in which orchids were the chosen crops (Figure 1.5, right) (Harvey and Zakutnyaya, 2011).

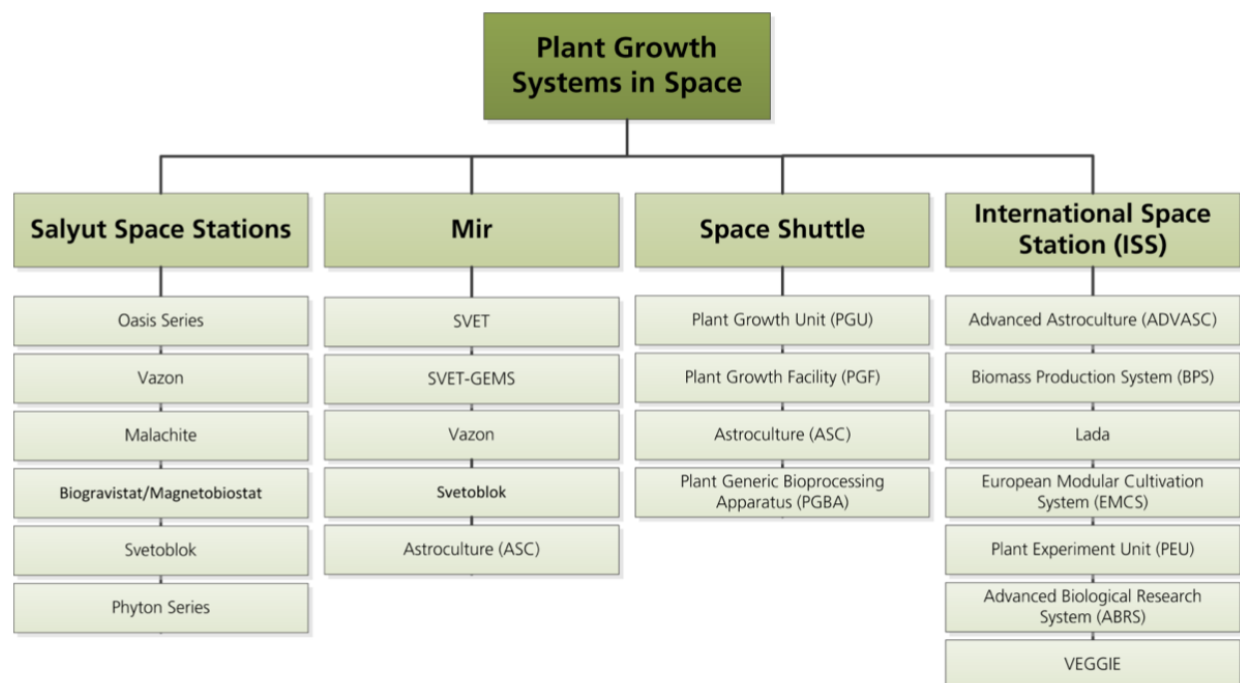


Figure 1. 4 Overview of plant growth chambers in space as described in Zabel et al. 2016.

Thereafter, Biogravistat and its upgrade, Magnetobiostat, were specifically designed to study the effects of microgravity. Later, Svetoblok represented the first system containing tomato plants in a sterile environment. Even if tomato plants grew slowly and no viable seeds were produced, this experiment demonstrated the possibility to reach, for the first time in space, the flowering of a crop species (Porterfield et al., 2003).



Figure 1. 5 Oasis 1M (left) and Malachite (right) plant growth chambers as exhibited in the Memorial Museum of Astronautics in Moscow (photos taken by M. Bamsey in 2014).

In 1982, the first seed-to-seed plant cycle was achieved using *Arabidopsis thaliana* plants on Phyton-3 growth system, equipped with an illumination source, an automated seed sowing machinery and a ventilation apparatus (Porterfield et al., 2003). During the following 30 years, several experiments were conducted on board the Mir, the Space Shuttle and the ISS. All these experiments contributed to several improvements on the hardware features (e.g., temperature and humidity control, illumination system and fluid delivery control), that led to the development of the VEGGIE Food Production System, launched in 2014 on board the ISS (Figure 1.6, left) (Stutte et al.; Massa et al., 2013). In August 2015, the four astronauts Yui, Kononenko, Lindgren and Kelly were officially allowed to eat their space-grown vegetables (Figure 1.6, right).



Figure 1. 6 VEGGIE prototype during the ground test (left); astronauts Yui, Lindgren and Kelly while they are eating self-grown lettuce on the ISS (right), ©NASA.

Also, ground simulation testbed for the setup of a greenhouse on the Moon and Mars have been carried on. Recently, the EDEN ISS (Evolution and Design of Environmentally-closed Nutrition-sources), was the first bio-regenerative greenhouse tested in Antarctica able to demonstrate that higher plants cultivation technologies are suitable for future long-term manned missions in confined environments (Figure 1.7) (Zabel et al., 2015; Imhof et al., 2018).

Furthermore, the University of Arizona developed a novel lunar habitat design, called Lunar Greenhouse (LGH) that can support the establishment of a lunar outpost (Figure 1.8) (Giacomelli et al., 2012).



Figure 1. 7 EDEN ISS greenhouse in Antarctica and its inside, ©DLR 2018.

Over the last years, the European Space Agency (ESA) has created the MELiSSA program (Micro-Ecological Life Support System Alternative) to develop a closed regenerative life system, using higher plants and micro-organisms processes to recycle resources (Wolff et al., 2014; Braun et al., 2018). Within the MELiSSA program, the state of knowledge concerning the impact of space environments on higher plants was analysed with the LiRHiPliSME project (Literature Review of Higher Plants in Space for MELiSSA).



Figure 1. 8 Prototype BLSS Lunar Greenhouse (LGH), © Giacomelli et al. 2012.

In the framework of these projects, and after over 40 years of space plant growth systems studies, the major candidate species were identified as lettuce, tomato, dwarf tomato, cucumber, bell pepper, watercress, red mustard, swiss chard, strawberry, radish, spinach, chives, basil, coriander and parsley (Zabel et al., 2016). Dueck et al., 2016 ranked the candidate species following the requirements of quality, yield and cultivation methods and the first six crops that met the criteria were lettuce, cucumber, dwarf tomato, chives, tomato and strawberry.

1.2 Plants survival in extreme conditions in space: the challenge of ionizing radiation

As previously introduced, the potential of plant survival and reproduction in space must consider conventional environmental parameters such as temperature, humidity, light, pressure, as well as specific space factors like microgravity, static magnetic fields (SMFs) and ionizing radiation (IR) (De Pascale et al., 2021a).

Plants are capable to adapt to several environmental conditions altering their physiology and their structure, but, the space environment is very unusual to plants. One of the main challenges of space exploration programs and plant growth and reproduction in space is represented by the effects of ionizing radiation (IR), a component of cosmic rays (GCRs). As an example, the IR levels on the Martian surface are due to GCRs and solar energetic particles (SEPs) (Chancellor et al., 2018). Both GCRs and SEPs can penetrate the Martian atmosphere and can produce secondary particles (e.g., neutrons and gamma rays) able to induce damages to the chemical

structure of the materials they pass through (Hassler et al., 2014). Conversely, on Earth, IR normally originates from geological processes and most studies regarding their effects on plants have been conducted under laboratory or highly controlled conditions (Caplin and Willey, 2018). In addition, extensive knowledge of radiation effects on biological entities was provided by the Chernobyl and Fukushima disasters (Mousseau and Møller, 2020).

1.2.1 Space ionizing radiation and their effects on biological entities

Due to the presence of both GCRs and SEPs, the radiation environment of deep space and low Earth orbit is very different from that on Earth's surface. GCRs generally consist of approximately 87% protons (i.e., hydrogen ions), 12% alpha particles (i.e., helium ions) and 1-2% heavier nuclei (e.g., lithium and nickel) (Chancellor et al., 2018). These ions are high-energy nuclei able to penetrate any shielding technology currently adopted on space vehicles. As described by De Angelis et al., 2007, GCRs originate from space and enter the Solar System, where they interact with the particles of the solar wind. SEPs comprise high-energy particles from the Sun originating by solar flares or coronal mass ejections (Takahashi et al., 2018). For example, the Martian atmosphere is much thinner than that of Earth and Mars lacks a global magnetic field to deflect energetic charged particles. These two main environmental factors lead to the presence of permanent ionizing radiation on Mars's surface. Despite space radiation research has expanded rapidly in the last years, large uncertainties remain in predicting and extrapolating data to radiation exposure in both humans and plants (Chancellor et al., 2018). Radiation dose is defined to be the energy (in Gray, Gy) deposited per unit mass in water. In general, the radiation dose in outer space and/or others planets is very difficult to calculate or predict. To this end, the HZETRN (High charge (Z) and Energy TRaNsport) NASA-JSC computer model has been used to calculate the GCR flux at various points in the solar system (John et al., 1995), considering also the effects of shielding. From calculation based on data from MOLA (Mars Orbiter Laser Altimeter) to determine altitudes (Zuber et al., 1992), the HZETRN predicted a dose range between 0.2-0.3 Gy/year on Mars surface, while the real dose-rate recorded in the first weeks of MARIE's (Mars Radiation Environment Experiment) operation in Mars orbit detected 0.3-0.4 Gy/year. MARIE was also able to record a SEP event in July 2002. During this event, MARIE trigger rate was so high that the data acquisition system was unable to keep it up, and the pulse-height data were unusable. Surprisingly, data inherent that SEP events were recorded by the GOES-8 satellite, revealing that the dose-rate reached a peak of about 10 mGy/day with a total SEP dose corresponding to about 30 mGy (Zeitlin et al., 2004). Despite many SEPs may show modest energy distributions, occasional and unpredictable high fluence

events could occur. For example, a particularly intense SEP happened in October 1989. It has been calculated to have delivered dose-rates as high as 1,454 mGy/hour to a possible exposed astronaut in a vehicle travelling in interplanetary space.

Data acquired from the Curiosity rover, using the adsorbed dose calculations made by the dosimeter RAD (Radiation Assessment Detector) in 2012-2013, allowed to calculate that, during a conventional 680 days space mission (i.e., 180 day-roundtrip travel, 500 days of continuative presence on the Mars surface at Gale crater), a living organism is exposed to a radiation dose of about 1.01 Gy (Hassler et al., 2014).

It has to be added that the fluence of GCR particles in interplanetary space fluctuates inversely with the solar cycle, with dose rates comprised between 50-100 mGy/year at solar maximum and between 150-300 mGy/year at solar minimum (Mewaldt et al., 2005). The fluence and occurrence of SEPs is unpredictable, but dose rates as high as 1,400-2,837 mGy/hour are possible (R.J. Michael Fry et al., 1989; Lawrence W. Townsend et al., 2006; Hu et al., 2009).

The International Commission on Radiological Protection (ICRP) established the 0.5 Gy/year dose limit for non-cancer risk in blood-forming organs. This dose limit is currently adopted by the European Space Agency (ESA), the Russian Space Agency (RSA) and the Japanese Space Agency (JAXA) to protect the health of space crews.

Considering these limits, the scenario currently favoured by NASA for future space programmes implying human crews, foresee a fast transit to Mars (4-6 months, one way) and a longer stay on the surface (18-20 months), with an estimated dose equivalent comprised between 0.68 and 1.36 Gy. These calculations were made considering the solar maximum, the solar minimum and the single SEP worst case occurred in 1972.

IR are the most prominent pro-oxidant stimuli that plants will have to cope with. IR may both directly damage the DNA structure and generate free radicals through the radiolysis process (Figure 1.9). Several studies have shown how IR can damage DNA directly (e.g., introducing double-stranded breaks, DSB) or indirectly via radiolysis, a chemical reaction that creates reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), superoxide anion (O_2^-), hydroxyl radicals ($\bullet\text{OH}$) and singlet oxygen (Figure 1.10) (Foyer and Noctor, 2016). Although there is significant literature concerning the effects of acute high dose rate exposures on plant genetics, growth, and development, much less is known concerning the effects of chronic low dose irradiation, especially those related to the impact of high energy protons and heavy ions (Mousseau and Møller, 2020). This is due to logistical challenges required to set up ground-based chronic exposure experiments. Conversely, studies regarding high acute doses have been conducted in the immediate aftermath of nuclear accidents or through controlled field

experiments using point sources and in a controlled laboratory experiments (Caplin and Willey, 2018).

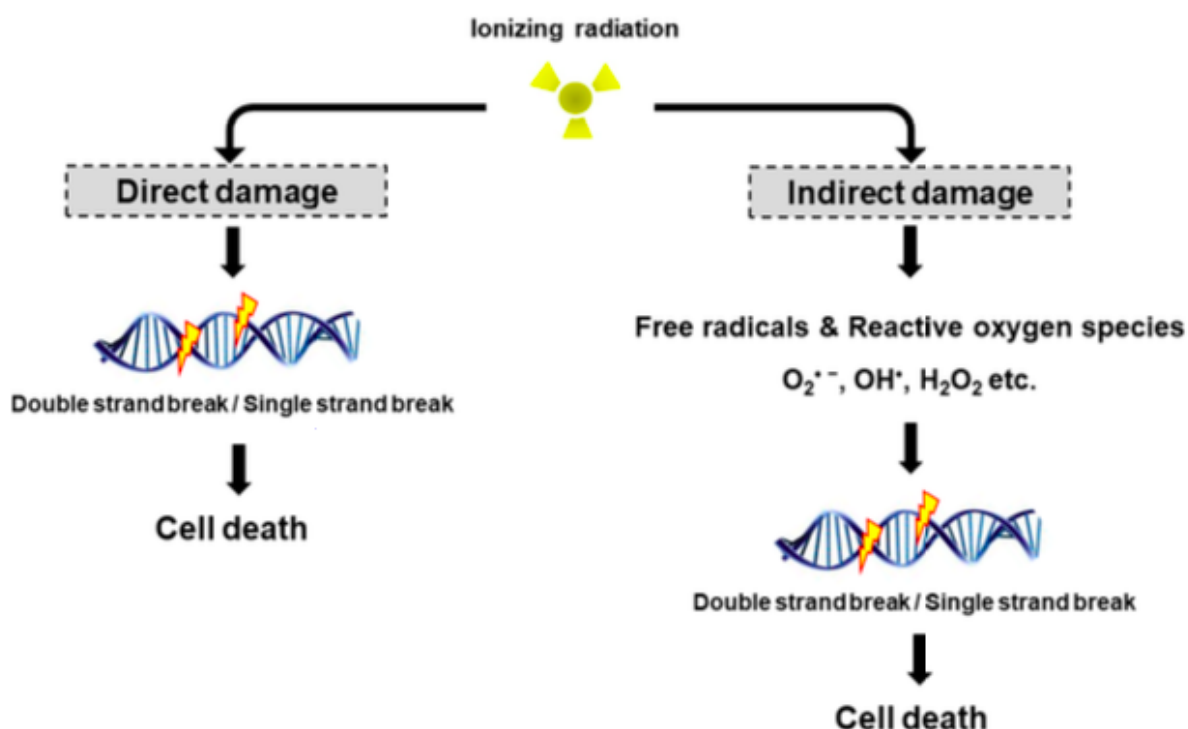


Figure 1. 9 Direct and indirect DNA damage by ionizing radiation. Radiation can directly interact with cellular DNA and cause damage. The indirect DNA damage caused by the free radicals is derived from the ionization or excitation of the water component of the cells, © Hur et al. 2017.

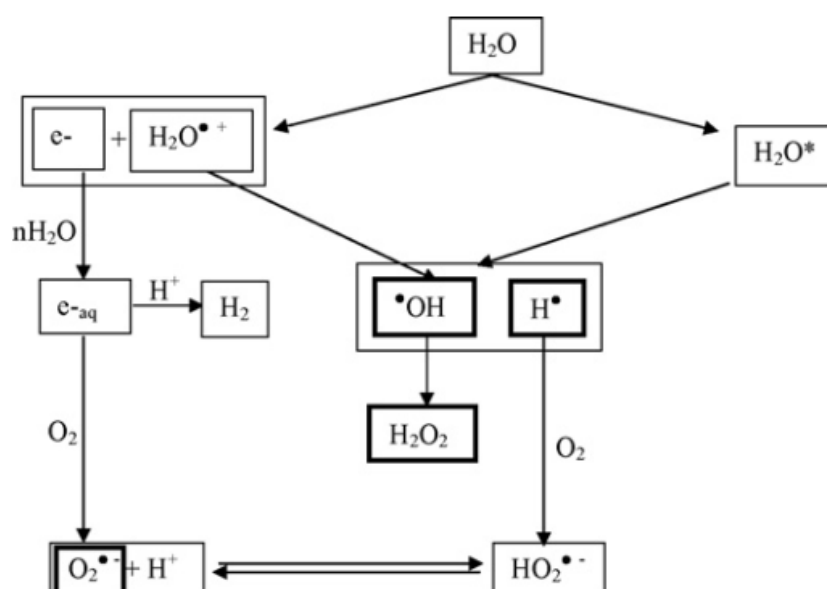


Figure 1. 10 Reactive oxygen species (ROS) produced by the ionizing radiation effects, ©Esanult et al. 2010.

Although the effects of cosmic radiation on plant development and reproduction are reported in literature, the majority of those experiments refers to acutely irradiated dry seeds and seedlings

and not to fully developed plants. As described by Esnault et al., 2010, IR can affect, especially at low doses, both cells and organisms at different levels, resulting in different responses due to individual phenotypic plasticity (Figure 1.11). In general, IR demonstrated to affect morpho-anatomical traits (e.g., cell wall structure, growth rate, reproductive success), genomic materials/proteins and the photosynthetic system, with consequences also on food quality (De Pascale et al., 2021a). Even if IR are calculated as an average (i.e., absorbed dose), irradiation hits will affect different areas of DNA, making difficult to draw a general pattern and to predict the resulting response (Kumagai et al., 2000; Maity et al., 2005; Kovalchuk et al., 2007; Esnault et al., 2010). Furthermore, the plant age and the time of radiation exposure may play an important role in integrating radiation effects (Kurimoto et al., 2010).

As an example, to understand the characteristics of IR-mediated mutations in different generations of rice after spaceflight, seeds of 11 varieties were carried by Shenzhou-3 spaceship in 2002 (Yu et al., 2007). On the M₁ generation, changes in agronomics traits including plant height, 1000-grain weight, panicle length and seeds per panicle, were observed. On the M₂ plant height and spike length were greater than control while in the M₃, the same two traits were positively or negatively affected in dependence of the individual plant considered. In particular, the observation that plant height changes in all three generations demonstrated that the mutation traits can be inherited to their progenies.

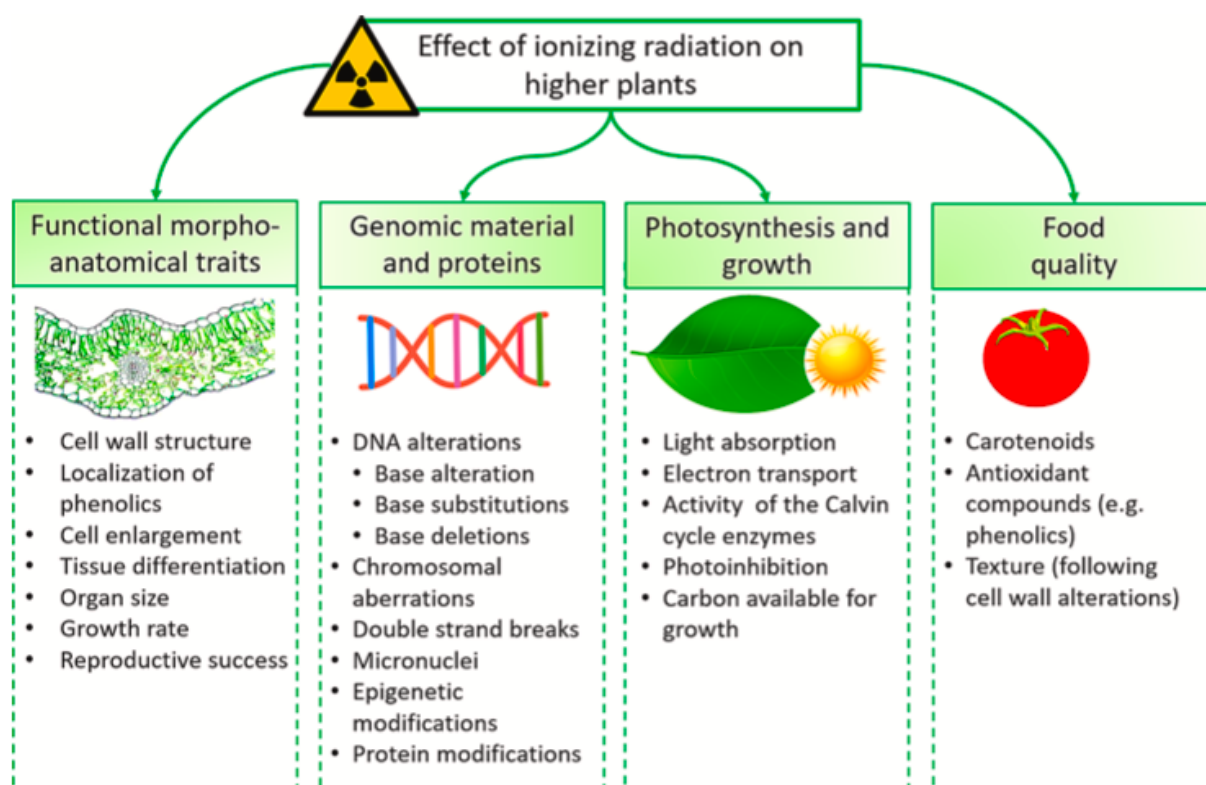


Figure 1. 11 Summary of the IR effects on plants as described in De Pascale et al. 2021.

In conclusion, the complexity of events implying IR and the difficulties in operating reliable calculation of corresponding radiation doses in outer space only allow, for the moment, to make predictions about the IR dose that both humans and plants will experience during a space mission.

1.2.1.1 The gamma radiation component of cosmic rays and its effect on biological entities

Biological stressors related to space radiation are due to the effects of linear energy transfer (LET) from a charged particle to the cell-matrix (Chancellor et al., 2018). To determine how quickly a particle loses energy when interacts with the matter, its charge, mass and energy must be considered. Indeed, heavier charged particles are capable to deposit a greater amount of energy per unit path length, resulting in a higher radiation-related risk, compared to lighter charged particles (Takahashi et al., 2018). Hence, in dependence on their LET, cosmic rays have been divided into high linear energy transfer (High-LET) (i.e., alpha particles, protons and neutrons) and low linear energy transfer (Low-LET) (i.e., gamma rays and X rays) radiations. There are four primary differences between gamma rays and X rays: source (i.e., gamma rays are emitted by the excited nucleus while X rays are emitted by the electrons outside the nucleus), frequency (i.e., gamma rays have frequencies above 10^{19} Hz while X rays frequencies vary from 10^{16} Hz to 10^{20} Hz), wavelength (i.e., gamma rays wavelength is smaller than that of the X rays) and photon energy. The latter describes the energy speed at which the rays are travelling. For example, ^{137}Cs radiation is characterized by an energy speed of 662 keV, ^{60}Co radiation by 2.5 MeV and X rays source radiation by 82 keV. This energy speed equates to penetration power and higher energies correspond to a higher penetration power. Due to these features, gamma rays are considered a highly penetrating IR, able to interact with matter through three mechanisms: photoelectric effect, Compton scattering and pair production. As a component of cosmic ionizing radiation and as already introduced, gamma-rays can damage DNA both directly and indirectly, and these damages appear to be dose-dependent. At high doses gamma rays generate permanent damages while decreasing the dosage, they can display a stimulatory effect (De Micco et al., 2011; Arena et al., 2014a).

To investigate plant responses to gamma rays, several experiments have been performed using Earth ground-based facilities on seeds and seedlings (Kim et al., 2004; Zaka et al., 2004; Maity et al., 2005; Kim et al., 2011; El-mahrouk and El-sheikh, 2013; Marcu et al., 2013a, 2013c; Fan et al., 2014; Van Hoeck et al., 2017). These studies showed that gamma rays affect plant morphology and physiology, with consequences on growth, development and reproduction

success, as well. Several components of the photosynthetic apparatus such as electron transport carriers, light-harvesting complexes, carbon reduction cycle enzymes and chlorophyll a and b were demonstrated to be sensitive to gamma rays (Kim et al., 2004; Marcu et al., 2013b, 2013c). More recently, several studies have been based on either chronic or acute exposure, with a focus on the effects of gamma rays on genome stability of *Arabidopsis thaliana* (Kovalchuk et al., 2000, 2007; Kumagai et al., 2000; Wi et al., 2007; Kurimoto et al., 2010; Kim et al., 2011; Qi et al., 2015). Exposure of seeds or seedlings to chronic doses from 0.1 Gy to 10 Gy of ^{60}Co gamma rays, increased homologous recombination frequency especially, in seedlings (Kovalchuk et al., 2000). Subsequently, Kovalchuk et al., 2007 profiled the global genome expression, confirming drastic differences in transcriptome between both chronically and acutely irradiated and not-irradiated *Arabidopsis* seeds. As described in Kovalchuk et al., 2000, the seeds germination rate was not affected by irradiation, while Kumagai et al., 2000 observed no differences in germination rate up to 1 kGy acute dose exposure. *Arabidopsis* seedlings exposed to 1-5 Gy acute doses of gamma rays developed normally, while the growth of plants irradiated with 50 Gy dose was significantly inhibited (Wi et al., 2007). In addition, the localization and accumulation of ROS, like H_2O_2 and peroxidase (POD), which represents the endogenous enzymatic defence of plant cells, varied depending on the cell and tissue type considered, with the parenchyma cells that resulted the most sensitive in *Arabidopsis*.

Over the last thirty years, besides *A. thaliana*, different plant species have been used for space cultivation tests. Among these, rice (*Oryza sativa*) is one of the most studied (Bayonove et al., 1984; Maity et al., 2005; Yu et al., 2007). For example, during the Biobloc III experiment conducted on the Salyut-7, samples of *Oryza sativa* cv. Cigalon and Delta were exposed to gamma rays at low acute doses (from 50 to 100 Gy) either before or after the spaceflight (Bayonove et al., 1984). During this experiment, preliminary results demonstrated that when rice seeds were treated with 50 Gy dose before launch there was a recovery from irradiation damage. Afterwards, Maity et al., 2005 reported that rice seeds irradiated in the lower acute dose range (50-150 Gy) resulted in a stimulatory effect on seedlings height, panicle length and seeds per panicle, with a significant decrease of these parameters when a higher dose range (200-350 Gy) was applied.

Recently, Marcu et al., 2013a, 2013c studied the gamma irradiation effects on *Zea mays* and *Lactuca sativa* demonstrating that the germination percentage and the germination index, as well as root and shoot lengths decreased by the increase of the acute dose irradiation (0.1-1 kGy and 0-30 Gy, respectively). In addition, while the amount of free radicals increased linearly on the

absorbed dose, an inverse relationship between the increasing irradiation dose and chlorophyll a, b and carotenoids was found.

Among other species irradiated with gamma rays, studies were conducted on *Pisum sativum*, *Curcuma alismatifolia*, *Jatropha curcas*, *Orthosiphon stamineus*, *Moluccella laevis*, *Lemna minor*, *Zizania latifolia*, *Abelmoschus esculentus* and *Paulownia tomentosa* (Zaka et al., 2004; Alikamanoğlu et al., 2007; Pick and Ling, 2008; El-mahrouk and El-sheikh, 2013; Fan et al., 2014; Taheri, 2016; Asare et al., 2017; Van Hoeck et al., 2017; Surahman et al., 2018).

Due to the different logistical challenges required to set up experiments with IR, all these studies were conducted using different radiation sources, doses, dose/rates and plant species. This scenario is made more complex considering how IR affect plant cultivar, age, organ or tissue and ploidy level. All these considerations make difficult to predict the resulting response and only a general pattern can be drawn: gamma rays generally affect plant morphology-related traits and the pigment content such as phenolic compounds, carotenoids and chlorophyll a and b (Kumagai et al., 2000; Maity et al., 2005; Kovalchuk et al., 2007; Esnault et al., 2010). More in general, plants experiencing chronic internal exposure to ionizing radiation exhibit a higher frequency of homologous recombination than acutely irradiated plants (Kovalchuk et al., 2000).

In addition and in conclusion, it has to be mentioned that, compared to the wealth of literature available in the area of irradiation tests on seeds and seedlings, information on radiation studies on whole and fully developed plants are currently lacking. Moreover, scarce information are currently available on the cumulative effects of IR on more than one generation of plants (Kordyum and Hasenstein, 2021).

1.2.1.2 The issue of counteracting a pro-oxidant environment for both humans and plants

The design of plant genotypes able to cope with non-terrestrial conditions is mandatory to overcome possible hindrances to crop cultivation in space environments for many reasons. As already introduced, due to cosmic radiation, pro-oxidant stimuli are the most prominent for plants artificially adapted to space agriculture. Acting directly and indirectly to delay oxidative damage, enzymatic players and metabolites activating endogenous antioxidant defence systems, may help plant growth in the space environment. In addition to plant survival, the issue of counteracting overproduction of free radicals generated by harmful ionizing radiation is crucial for human survival in space outposts, as well, and opens the way to the ideal ‘anti-oxidant space fresh food’. Indeed, the oral intake of clinically tested chemical radioprotectants (i.e., thiols, aminothiols, thiadiazoles and benzothiazoles) is limited due to toxicity (Copp et al., 2013). Therefore, there is

great interest in the development of plants able to accumulate natural anti-oxidants that can be possibly introduced with the diet (Gómez et al., 2021).

1.2.1.3 Other stresses of a space-related confined environment

Together with the above-mentioned abiotic ‘macro-stresses’ related to IR and pro-oxidant stimuli, plants intended for space agriculture will have to cope with a plethora of abiotic ‘micro-stresses’ related to the different cultivation environment/systems that will be adopted. Furthermore, plants will have to share with human future crewed space habitats (spaceflights, planetary outposts and life-support systems) where stringent microbial quality requirements may not be achieved, exposing plants to contaminations possibly leading to plant diseases and spoiling of fruits (Amalfitano et al., 2020).

The ‘design’ of plant ideotypes suitable to these environments may be achieved by further refinement of existing biochemical and physiological features, such as specialized metabolites, that plants use to survive stress. Novel genotypes boosting biosynthetic pathways for the production of specialized metabolites can be generated by manipulation of their regulators.

1.3 Tomato as an ideotype for space cultivation

Due to their prominent position as commodities, plants of the *Solanaceae* family are considered as the most interesting genotypes to be manipulated to obtain possible ideotypes for agrospace applications able to integrate astronauts’ diet currently based on pre-packaged, or dried food with the inconvenience that critical nutrients may be lost (Cooper et al., 2017). In addition, as already mentioned, these plants have already provided insights into biological processes related to space stimuli exposure. As initial examples, concerning exposure to IR, pepper and potato (*Capsicum annuum* and *Solanum tuberosum*, respectively) have been subjected to gamma irradiation (Kim et al., 2004; Cheng et al., 2010). The acute irradiation of pepper with a dose range between 2 and 16 Gy did not induce significantly different photosynthetic efficiency (Fv/Fm) and maximum quantum yield of Photosystem II (PSII) (Y_{Fm}) (Kim et al., 2004). Meanwhile, minitubers of potato cv. Shepody were acutely irradiated with doses ranging from 0 to 80 Gy showing that the emergence of tubers cannot be promoted over the 60 Gy dose (Cheng et al., 2010).

Tomato (*Solanum lycopersicum* L.) is one of the most cultivated vegetables worldwide and its fruits are the largest dietary source of lycopene, ascorbic acid, alpha-lipoic acid, choline, folic acid and lutein (Bognar et al., 2013; Mirdehghan and Valero, 2017). Tomato is one of the model systems for *Solanaceae* family, providing new insights into biological processes that cannot be obtained using other model plants such as *Arabidopsis thaliana*.

As described by Dueck et al., 2016 dwarf tomato is the fourth crop that meets the specific requirements for space cultivation due to its short life cycle (i.e, 90-100 days from sowing to fruit ripening) and the small size (i.e, approximately 15 cm high at maturity). Spencer et al., 2019 identified three dwarf tomato cultivars (i.e., Mohamed, Red Robin, Sweet N' Neat) as space candidate crops. Another dwarf determined growth tomato cultivar, MicroTom, has been used by Colla et al., 2007, De Micco et al., 2014 and Arena et al., 2019 to study, respectively, the effects of microgravity, high-LET and low-LET ionizing radiation on edible plants. MicroTom was initially designed as an ornamental plant by conventional breeding between the cv. Florida Basket and cv. Ohio 4013-3. It carries the two mutations *self-pruning* (*sp*) and *dwarf* (*d*) (Scott and Harbaugh, 1989). In addition, MicroTom is an autogamous crop that, under an appropriate treatment with N-1-naphthylphthalamic acid (NPA, an inhibitor of the auxin transport), can produce fruits without the need of pollination (Serrani et al., 2010). Among the relevant features of this cultivar, there is very good adaptability to growth in closed, confined environment, under fluorescent or LED lighting and in hydroponic systems, making this plant an ideotype for space cultivation (Arena et al., 2019b).

Due to all these features amenable to agrospace applications, tomato has been tested for response to astrophysical stimuli. Studies on the influence of long-term exposure to space flight were conducted on tomato seeds flown for 6 years onboard the space station MIR (Nechitailo and Jinying, 2005). This chronic exposure to space environment led to high variability in growth, developmental parameters and yield, as an adaptive response of cells to alterations of their environment (Nechitailo and Jinying, 2005). Marzioli et al., 2020 proposed MicroTom inside a dedicated 12 U CubeSat while MicroTom hairy roots have been already tested under static magnetic fields, X-rays and gamma rays exposure (Villani et al., 2017 and Desiderio et al., 2019). Concerning IR, tomato seeds were subjected to low-LET and high-LET acute radiation from X-rays and Ca heavy ions, respectively, with no significant differences observed in the germination rate (De Micco et al., 2014; Arena et al., 2019b). While both low-LET and high-LET differently affected growth parameters such as plant height, leaf number and leaf area, the low-LET irradiation had no detectable effects in fluorescence parameters (i.e., quantum yield of PSII electron transport and non-photochemical quenching) and did not affect total photosynthetic pigment content, demonstrating that this type of irradiation was not capable to alter the photosynthetic apparatus efficiency (De Micco et al., 2014). As said, novel genotypes boosting biosynthetic pathways for the production of specialized metabolites counteracting free radicals generated upon IR may be generated by manipulation of the regulators of these pathways. Among the regulators of these plant specialized metabolites, there are MYB transcription factors, known

for their contribution to the increased complexity of land plants and to be regulators of plant responses to the environment (Lloyd et al., 2017). Members of this large family of regulators are key players in the modulation, among others, of the biosynthesis of flavonoids, like anthocyanins, in different plant parts and response to all kinds of stimuli (Ambawat et al., 2013). Despite the richness in valuable compounds known for their nutraceutical effects, accumulation of flavonoids in tomato fruits is sub-optimal and, with some exception, anthocyanins, widely known because of their potent antioxidant properties, are not present (Mes et al., 2008). Due to these properties, anthocyanins have increasing applications in the food sector and there is also growing interest in the design of food crops with improved levels and composition of these antioxidant nutraceuticals for ‘agrospace’ applications (Bijlani et al., 2021). Agrospace crops, among which tomato, are, therefore, candidates for the application of technologies aiming to improve both their content in anti-oxidant nutraceuticals for space crews survival and their resistance to biotic and abiotic stresses (Zabel et al., 2015).

1.3.1 Anthocyanins functions and antioxidant properties

Anthocyanins are water-soluble flavonoids deriving from the phenylpropanoid pathway, that are usually present in different plants tissues, inside the vacuoles (Koes et al., 2005). Anthocyanins are the glycosylated forms of anthocyanidins and are generally formed by a flavylum cation backbone hydroxylated in different positions (Figure 1.12) (Mattioli et al., 2020).

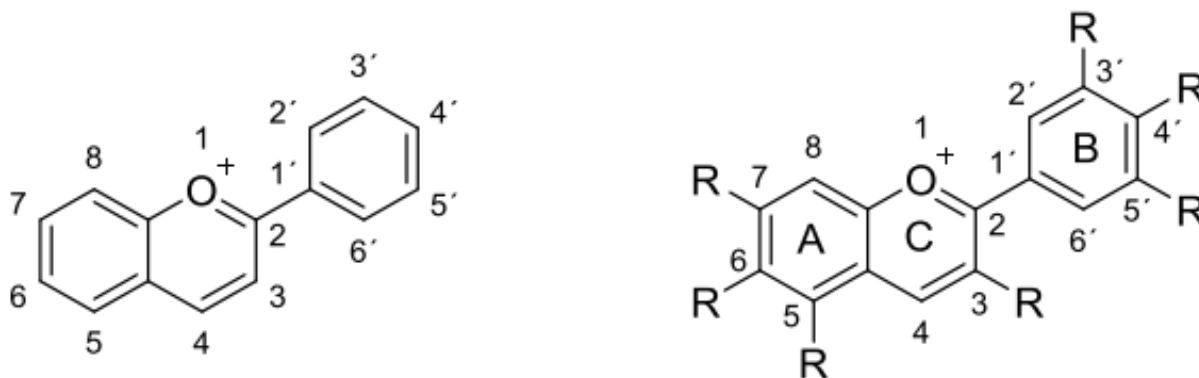


Figure 1. 12 Chemical structure of the flavylum cation (left) and of anthocyanidin backbone (right), ©Mattioli et al. 2020.

Anthocyanins are unique in terms of stability and their capacity to change structure following changes in the pH value. For example, at pH 1-3 they are red and form the most stable structure (i.e., flavylium cation form) while at pH 5 they reach the colourless carbinol pseudo base that could turn into a chalcone. Finally, at pH 7-8 they form a blue-purple quinoidal base, whereas at pH 11 an unstable dianionic blue-green form is generated (Figure 1.13) (Pojer et al., 2013).

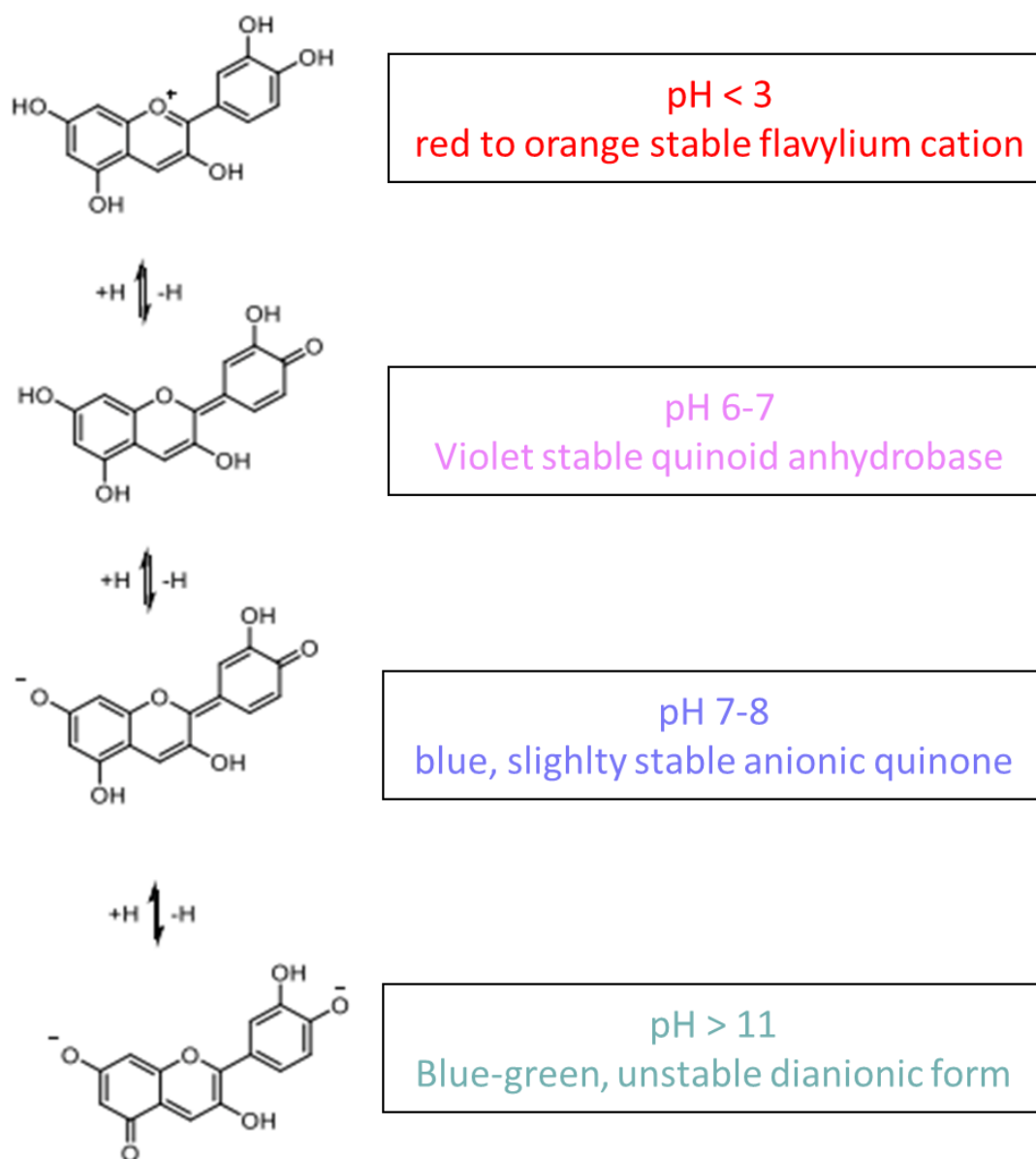


Figure 1. 13 Molecular stated and electronic delocalization of cyanidin at different pH values, □Mattioli et al. 2020.

As previously said, the presence of anthocyanins can be detected in different plant parts such as stem, leaves and storage organs (Tena et al., 2020). Normally, the fruits with the highest content of anthocyanins are berries, grapes, currants and some tropical fruits, with the group of edible vegetables, leafy vegetables, grains and tubers that showed the highest concentration (Tena et al., 2020). Over 700 anthocyanins have been identified in nature (Andersen and Jordheim, 2013) and the most common are derivatives of six anthocyanidins, namely delphinidin, peonidin, petunidin, malvidin, cyanidin and pelargonidin (Figure 1.14) (Gao et al., 2018).

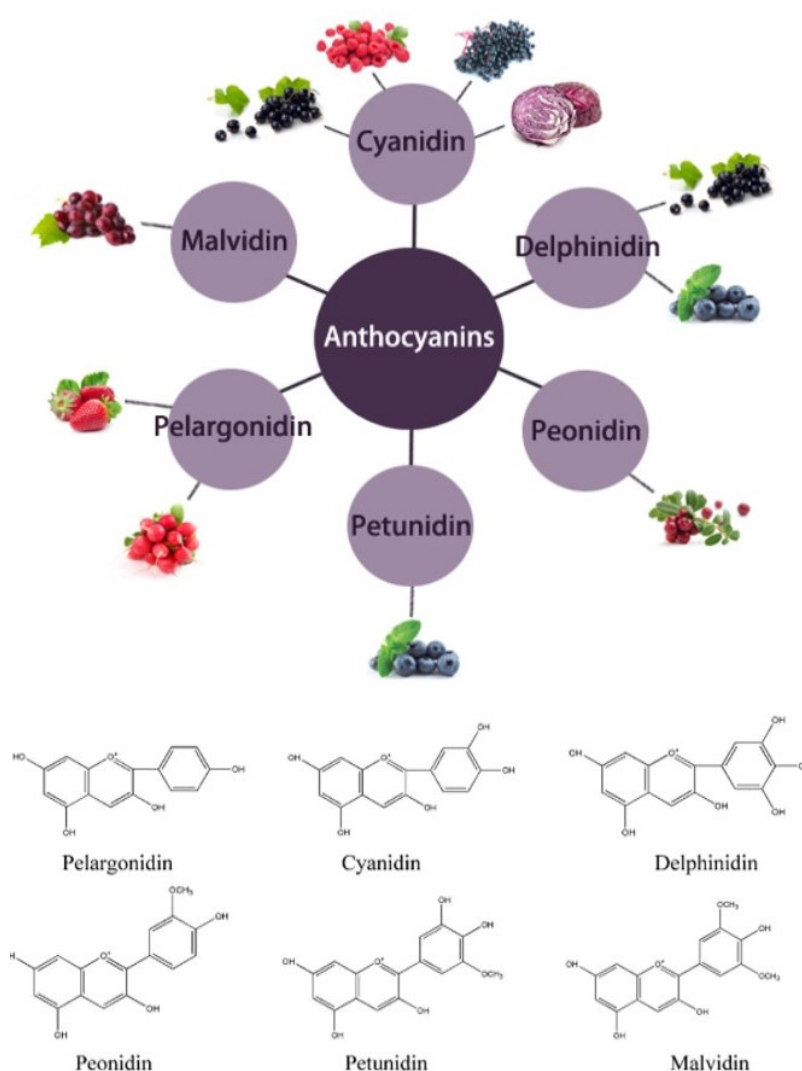


Figure 1. 14 Anthocyanins identified in different fruits, vegetables, and edible flowers, ©Kristo et al. 2016.

Several environmental stimuli, such as biotic and abiotic stress can induce the accumulation of anthocyanins, which are generally involved in the modulation of ROS-pathway, due to their high capacity of acting as scavengers for radical species (Gould et al., 2002; Tena et al., 2020). It is commonly accepted that phenylpropanoids contributed to the early land plant adaptation to terrestrial stress (Graham et al., 2004). Gould et al., 2002 demonstrated that under conditions of

low temperatures and/or strong light, red leaves are often photo-inhibited less than their acyanic counterparts and this difference can be ascribed to the absorption by anthocyanins of quanta that would otherwise damage the chloroplasts. Indeed, flavonoids can absorb the most energetic solar wavelengths (i.e., UV-B and UV-A), inhibit the generation of ROS and quench them once they are formed (Agati et al., 2012; Brunetti et al., 2013). Anthocyanins in the vacuole of mesophyll cells are generally present in high concentration and hence capable of removing H₂O₂ freely diffusing out of the chloroplast or the peroxisomes, under severe light stress and when catalase (CAT) or ascorbate peroxidase's (APX) activity is depressed (Ferrerres et al., 2011; Brunetti et al., 2013). It has to be noted that ROS production is also linked to hormone action for a wide range of plant responses to developmental and environmental processes, leading to growth and acclimation responses (Foyer and Noctor, 2016). Generally, plant cells can use ROS in signal transduction pathways to initiate processes like growth and acclimation (Foyer and Noctor, 2016).

Nowadays, anthocyanins and their food derivatives have become popular due to their benefits for human health (e.g., reduced risk of inflammatory processes, cancer, neurodegenerative and cardiovascular diseases) (Figure 1.15) (Lila, 2004; Pojer et al., 2013; Salehi et al., 2020). Tena et al., 2020 summarized the health benefits of anthocyanins in respect to eye health (e.g., protective effect during retinal inflammation, increase of ocular blood flow, prevention of retinal degeneration), cardiovascular diseases (e.g., in vitro antithrombotic properties, vasorelaxation properties, decrease of susceptibility to ischemia-reperfusion injury and infarct size, improvement of lipid profile and platelet function), antiobesity effects (e.g., reduction of sugar concentration in urine and plasma in rats, suppression of fat tissue gain, upregulation of adipocytokine secretion and gene expression in rat), antimicrobial effects (e.g., inhibition of gram-negative bacteria, induction of cell damage by destroying the cell wall, the membrane and intracellular matrix), antidiabetic effects (e.g., amelioration of hyperglycaemia and insulin sensitivity, alleviation of glomerular angiogenesis of diabetic kidneys in mice, amelioration of renal apoptosis in diabetic nephropathy mice).

Despite the well-known effects on human physiology, a generally recognized poor half-life of anthocyanins due to partial ability to cross plasma membranes, sensitivity to pH, digestive enzymes, microbiota and biliary acids, represent a possible hindrance to the full efficacy of these molecules (Tena et al., 2020). Moreover, the mechanisms through which anthocyanins exert their bioactivity functions are not yet fully understood.

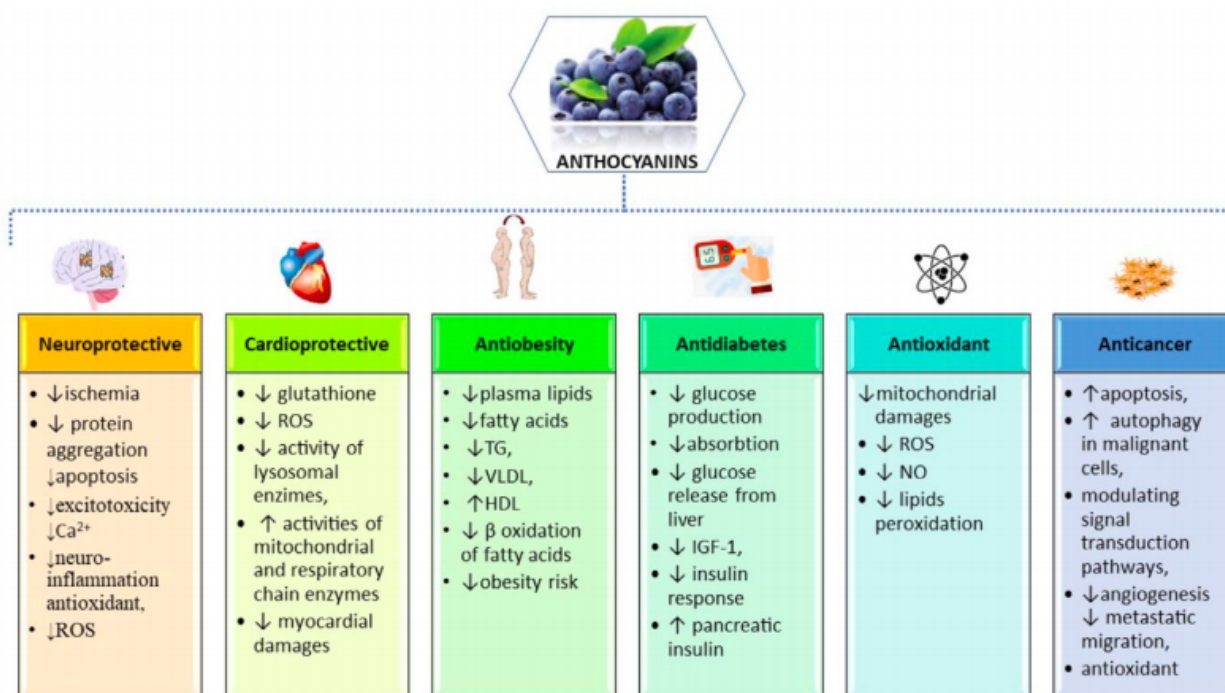


Figure 1. 15 Scheme of the most important pharmacological properties and molecular mechanisms of action of anthocyanins, ©Salehi et al. 2020.

1.3.2 Anthocyanins biosynthetic pathway

Anthocyanins biosynthesis is part of the larger phenylpropanoids pathway, which, starting from the aromatic amino acid phenylalanine, produces a range of secondary metabolites (Holton and Cornish, 1995). The anthocyanins biosynthetic pathway has been extensively studied in several plant species, including *Petunia hybrida*, *Antirrhinum majus* and *Zea mays* (Quattrocchio et al., 1993; Koes et al., 2005; Fang et al., 2019). Indeed, the analysis of the regulation of flavonoid genes in flowers of both *Petunia* and *Antirrhinum*, indicates that these genes are temporally and spatially regulated and their modulation also depends from light, temperature and biotic/abiotic stresses (Petroni and Tonelli, 2011). In general, the flavonoid genes have been classified as either early or late flavonoid biosynthetic genes (EBGs and LBG, respectively) (Quattrocchio et al., 1993).

The shikimate/arogenate pathway and the tricarboxylic acid cycle (TCA) provide the key flavonoid precursors phenylalanine and malonyl-CoA (Figure 1.16).

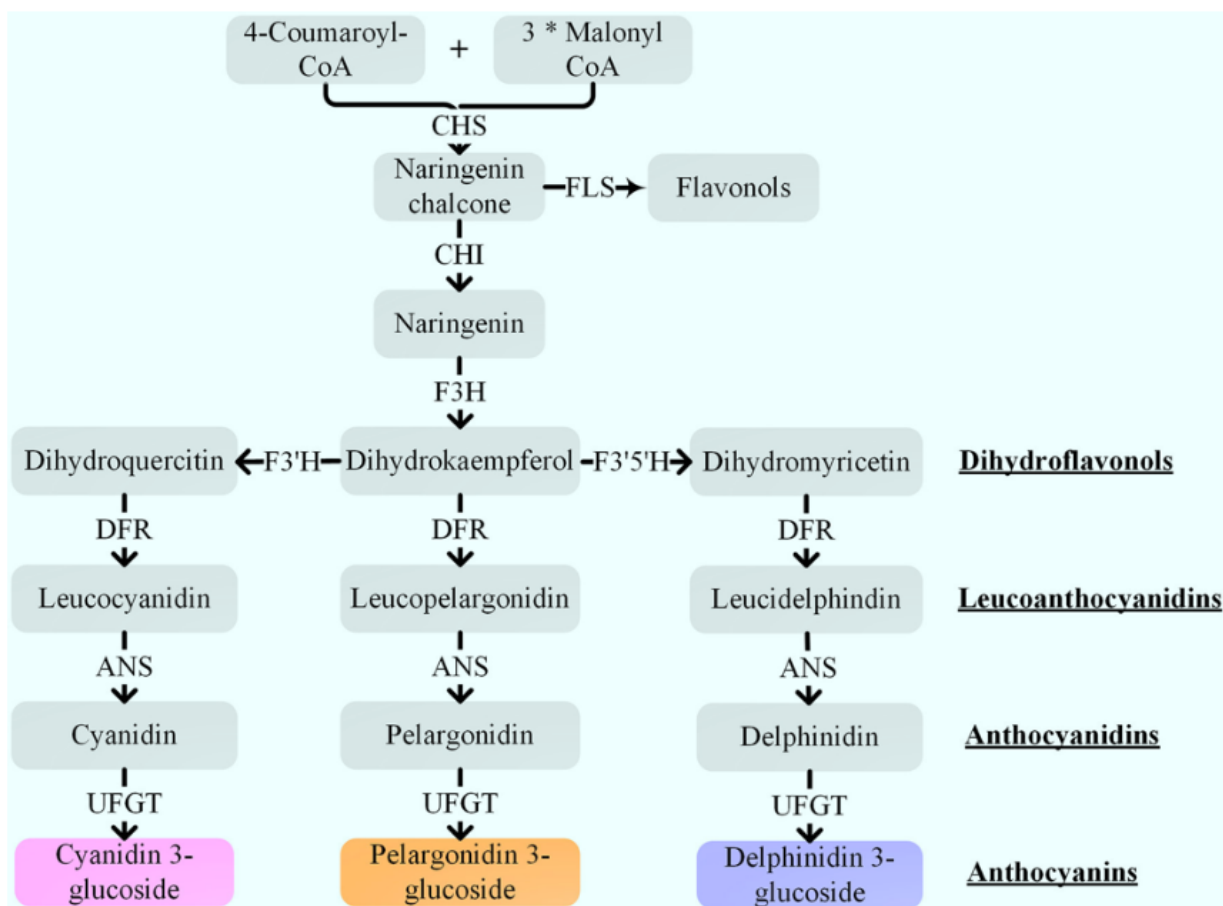


Figure 1. 16 General scheme of phenylpropanoid biosynthetic pathway, ©Liu et al. 2018.

Chalcone synthase (CHS) catalyses the stepwise condensation of three acetate units from malonyl-CoA with p-coumaroyl-CoA to yield tetrahydroxychalcone (naringenin chalcone). The chalcones are the first coloured flavonoid and provide yellow colouration to petals (Gould et al., 2009). Chalcone isomerase (CHI) then catalyses the stereospecific isomerization of the yellow-coloured chalcones to the colourless naringenin. Naringenin is converted into the three flavanones dihydrokaempferol (DHK) by flavanone 3-hydroxylase (F3H), pentahydroxylflavanone by flavonoid 3',5'-hydroxylase (F3'5'H) and eriodictyol by flavonoid 3'-hydroxylase (F3'H). Subsequently, DHK can be either hydroxylated by flavonoid 3'-hydroxylase (F3'H) to produce dihydroquercetin (DHQ) or by flavonoid 3',5'-hydroxylase (F3'5'H) to produce dihydromyricetin (DHM). The next step in the pathway is the conversion of these dihydroflavonols into flavan-3,4-cis-diols (leucoanthocyanidins) by dihydroflavonols 4-reductase (DFR) or into flavonols by the flavonols synthase (FLS). In the last part of the anthocyanin biosynthetic pathway, further oxidation, dehydration and glycosylation of the different leucoanthocyanidins produce the corresponding brick-red pelargonidin, pale-red cyanidin and blue delphinidin (Holton and Cornish, 1995).

1.3.3 Transcription factor-mediated control of anthocyanins biosynthesis

Most of the regulation of anthocyanins biosynthesis occurs via coordinated transcriptional control of structural genes, as extensively described by Koes et al., 2005 and recently summarized by Liu et al., 2018 and Zhang et al., 2019. This regulatory system involves a transcriptional activation complex known as MBWW, consisting of R2R3-MYB transcription factors, basic Helix-Loop-Helix (bHLH) and WD Repeat Proteins (WDR) (Koes et al., 2005; Quattrocchio et al., 2006). This complex is also boosted by the presence of a WRKY transcription factor, which confers specificity for target genes involved, among others, in vacuolar hyperacidification (Verweij et al., 2016). The WD40 is a WDR protein expressed ubiquitously, including several tissues that lack anthocyanins, indicating that this protein is not the limiting factor in anthocyanins biosynthesis (Koes et al., 2005). By contrast, the expression of bHLH and MYB factors is confined to pigmented tissues (Passeri et al., 2016). In general, MYB activators are mainly from the R2R3-MYB type, while repressors consist of both R2R3 and R3-MYB transcription factors. As reported by Quattrocchio et al., 2006, a specific subgroup of R2R3-MYB genes (which includes maize *Cl*, petunia *AN2* and *Arabidopsis PAP1/PAP2*) are not able to function on their own and require the presence of a member of the R/B family of bHLH coactivators. Specifically, the physical interaction between R2R3-MYB and bHLH is mediated by the R3 repeat and the N-terminal region of the bHLH factor. Therefore, in *Petunia* two bHLH proteins, AN1 and JAF13 (Spelt et al., 2000), are known to interact with AN2 to regulate LBGs genes (Quattrocchio et al., 1999). AN1 requires the physical interaction with AN2 (expressed in petal limb) or AN4 (expressed in petal tube and anthers) to activate biosynthetic genes such as *DFR* (Quattrocchio et al., 2006).

Recently, both Koes et al., 2005 and Liu et al., 2018 hypothesized a model that describes the regulatory mechanism of anthocyanins biosynthesis (Figure 1.17).

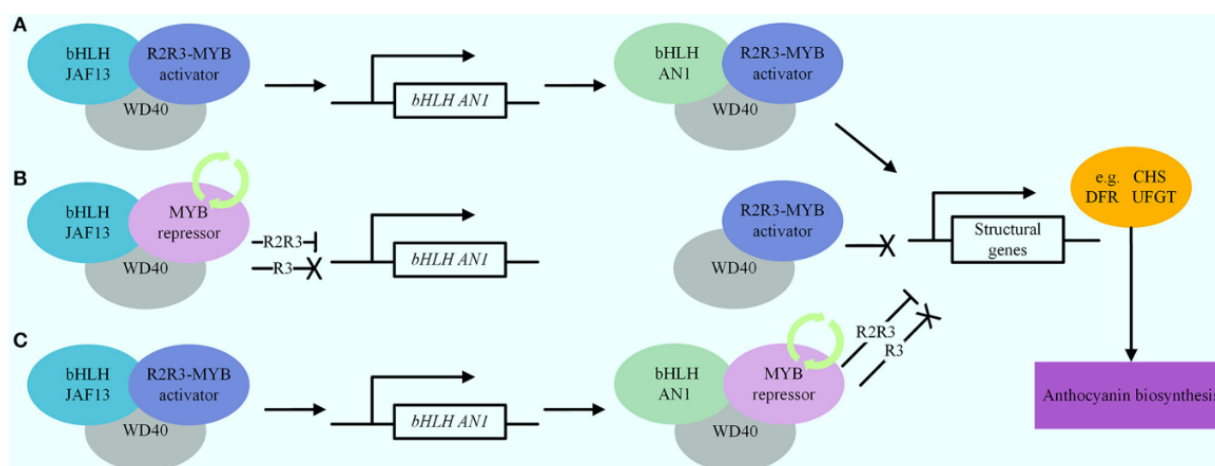


Figure 1. 17 Model depicting the role of MYB, bHLH and WD40 regulators in the modulation of the anthocyanin biosynthetic genes, ©Liu et al. 2018.

First, the R2R3-MYB transcription factor interacts with the bHLH JAF13 and WD40 protein forming the MYB-JAF13-WD40 complex, which activates the transcription of bHLH *AN1*. Subsequently, the R2R3-MYB binds to AN1 and WD40 forming the MYB-AN1-WD40 complex that can positively regulate the anthocyanins biosynthesis (Figure 1.17A). By contrast, the repression of anthocyanins biosynthetic genes is caused by MYB repressors that compete with the MYB activators to bind to JAF13. In this case, an inactive R3-MYB-JAF13-WD40 complex, unable to upregulate the expression of *AN1*, is formed (Figure 1.17B). Alternatively, the anthocyanins downregulation could occur when MYB repressors compete with MYB activators for binding AN1 in the MYB-AN1-WD40 complex (Figure 1.17C).

1.3.4 Enhancing anthocyanin biosynthesis in tomato

As previously reported, flavonoids accumulation in tomato fruits is sub-optimal and, with some exceptions, anthocyanins are not produced (Jones et al., 2003; Mes et al., 2008). For these reasons, over the last decades, several attempts to improve anthocyanins content in tomato have been conducted, reporting anthocyanin accumulation in fruits and other organs (Colanero et al., 2020)(Bovy et al., 2002; Mathews et al., 2003; Butelli et al., 2008; Mes et al., 2008; Povero et al., 2011; Schreiber et al., 2012; Kiferle et al., 2015; Su et al., 2016; Scarano et al., 2018; Blando et al., 2019; Jian et al., 2019).

The combined overexpression of the transcription factor MYB-type *Cl* and MYB-type *Lc* in fruit of tomato plants resulted in a strong induction of flavonoid biosynthesis in the flesh of the fruit, while anthocyanins accumulated only in vegetative tissues and occasionally in young immature fruits (Figure 1.18A) (Bovy et al., 2002). Mathews et al., 2003 studied the effect of the *ANT1* gene on wild type tomato *Solanum chilense*, obtaining purple vegetative tissues and purple spotted fruits (Figure 1.18B). As described by Mathews et al., 2003 and Schreiber et al., 2012, the *ANT1* overexpression caused the upregulation of genes that encode proteins in both early and later steps of anthocyanidin biosynthesis, as well as genes involved in the glycosylation and transport of anthocyanins into the vacuole (Figure 1.19).

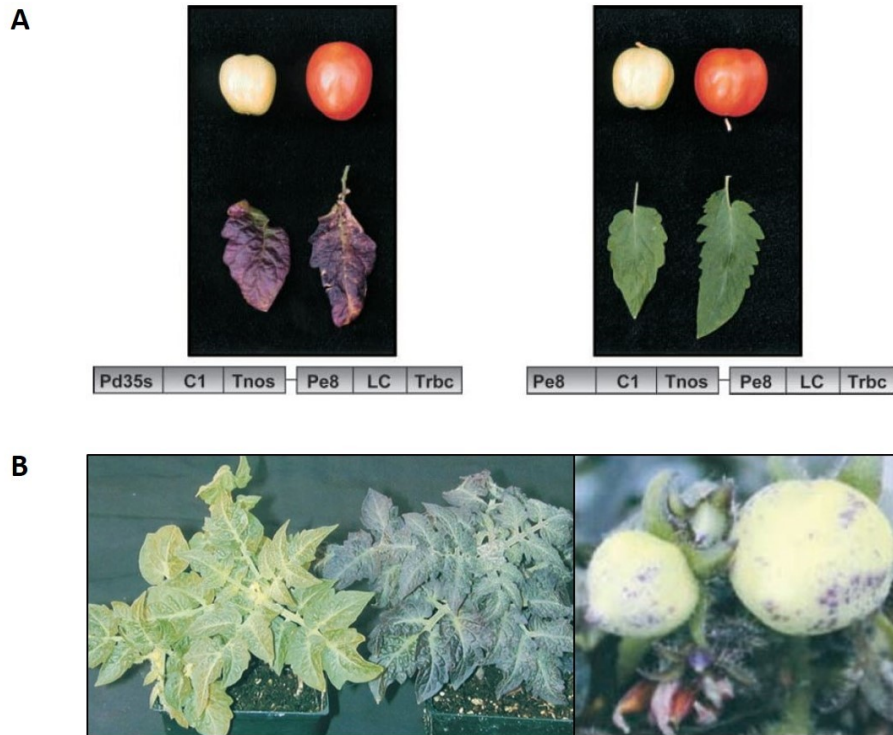


Figure 1. 18 (A) Phenotypal analysis of leaves, green and red fruits of LC/C1 plants, ©Bovy et al. 2002. (B) Wild type and *ant1* mutant plant of T1 (left); purple spotted fruits (right), © Mathews et al. 2003.

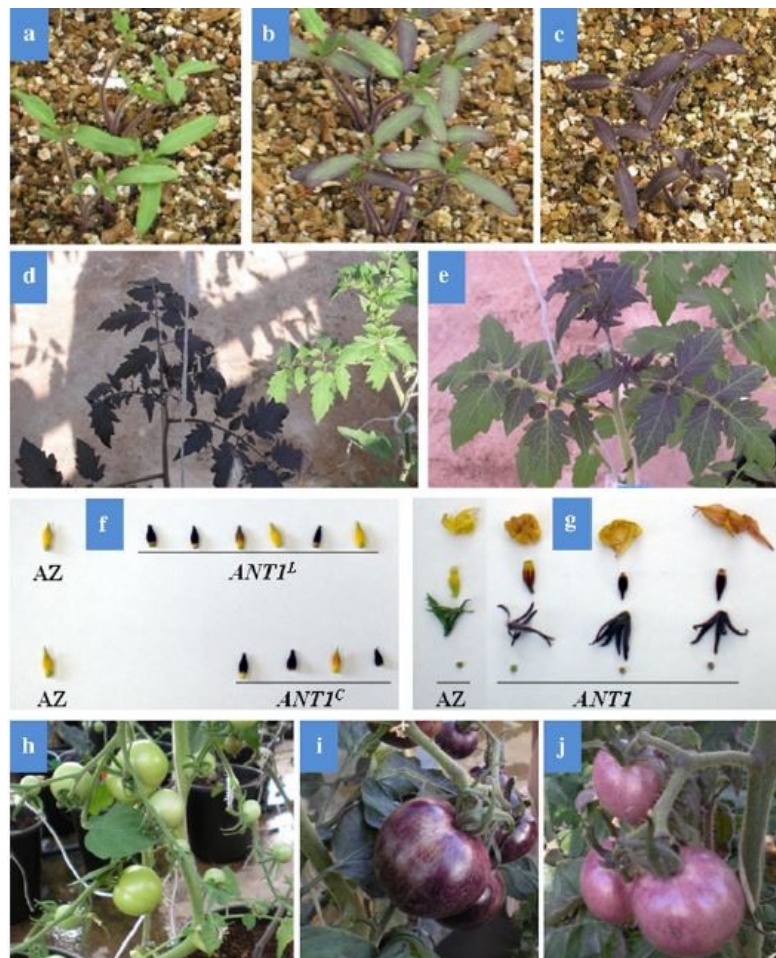


Figure 1. 19 Phenotype of transgenic tomato plants overexpressing ANT1 in leaves (b, c, e), flowers, petals, sepals, anthers, ovaries (f) and fruits (i, j). Phenotype of wild type leaves, reproductive organs and fruits were reported in a, d, f, g, h, ©Schreiber et al. 2012.

In addition, the ectopic expression of *ANT1* and *SIAN2* resulted sufficient to activate the whole anthocyanin biosynthetic pathway (Figure 1.20A) (Kiferle et al., 2015) while the introgression in domesticated tomato of two loci, *Aft* and *atv*, was found to generate the anthocyanin petunidin-3-(p-coumaryl)-rutinoside-5-glucoside in fruit epidermis (Figure 1.20B) (Mes et al., 2008; Povero et al., 2011; Blando et al., 2019).

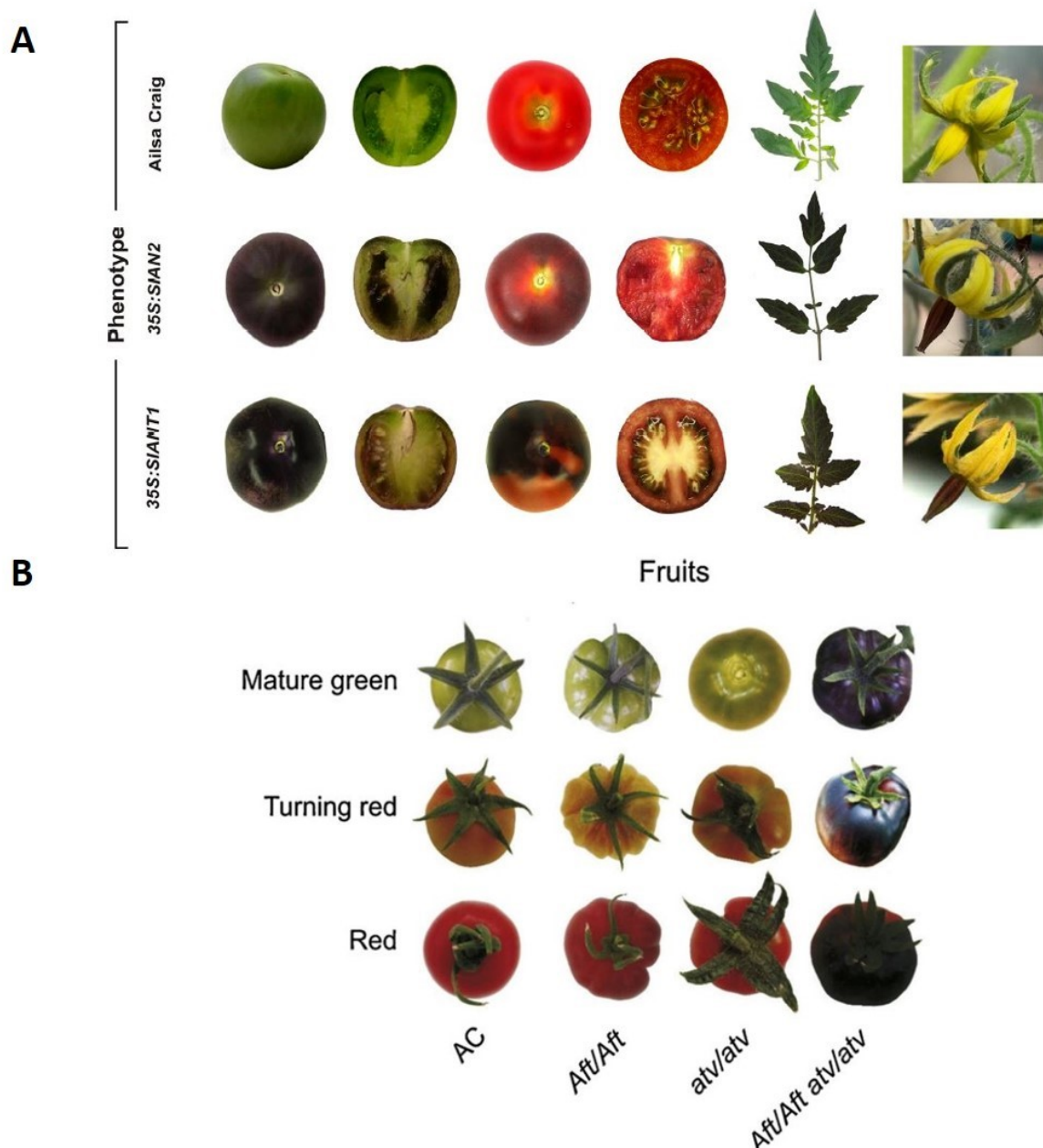


Figure 1. 20 (A) Phenotype of transgenic lines SIAN1 and SIAN2 compared to control plants (Alisa Craig), ©Kiferle et al. 2015. (B) Photographs of mature green, turning red and red tomato fruits from Alisa Craig (AC), Aft/Aft, avt/atv and Aft/Aft avt/atv plants, ©Povero et al. 2011.

The greatest enrichment in tomato anthocyanins has been reached by Butelli et al., 2008 through the expression of an R2R3-MYB protein (Rosea) and a bHLH protein (Delila) from snapdragon (*Antirrhinum majus*), under the control of fruit specific tomato promoter E8 (Figure 1.21A). Nevertheless, it has to be noted that in this work the most interesting bioaccumulation anthocyanins level (i.e., 2.83 ± 0.46 mg/g fresh weight) was achieved in hemizygous line N and not in a pure homozygous line. This engineered tomato was, therefore, used by Scarano et al., 2018 to create the ‘Bronze’ tomato, a plant co-expressing different transcription factors (i.e., *Del/Ros*, *MYB12* and *StSy*) and able to accumulate anthocyanins, flavonols and resveratrol in fruits (Figure 1.21B).

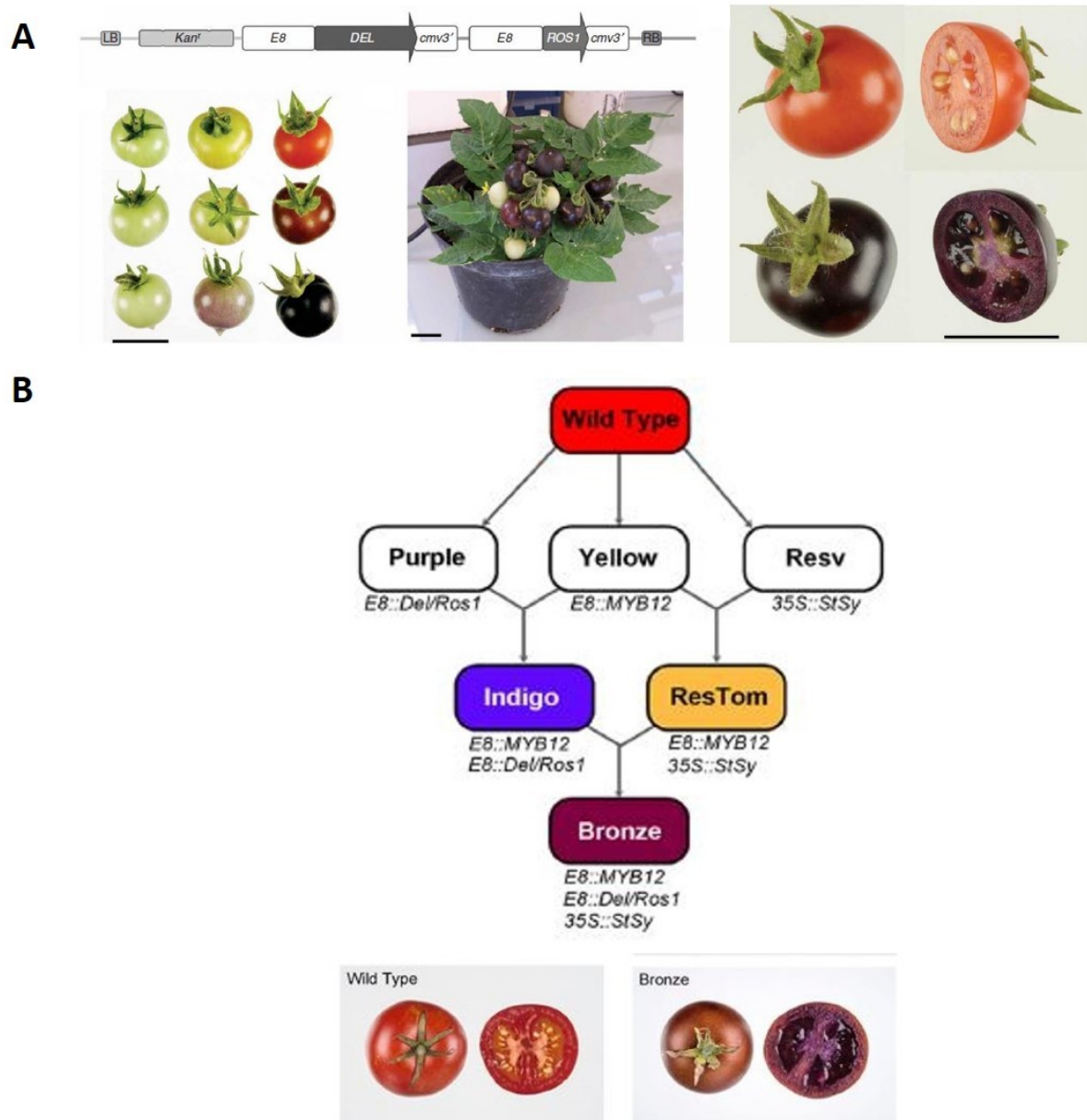


Figure 1. 21 (A) Phenotype of T1 generation tomatoes expressing both del and Ros1 under the control of E8 promoter, ©Butelli et al. 2008. (B) Breeding strategy adopted to develop the Bronze tomato, ©Scarano et al. 2019.

Interestingly, Jian et al., 2019 obtained anthocyanins accumulation, as well as an enhancement of volatile aroma compounds, with the single use of *SLAN2* (Figure 1.22).

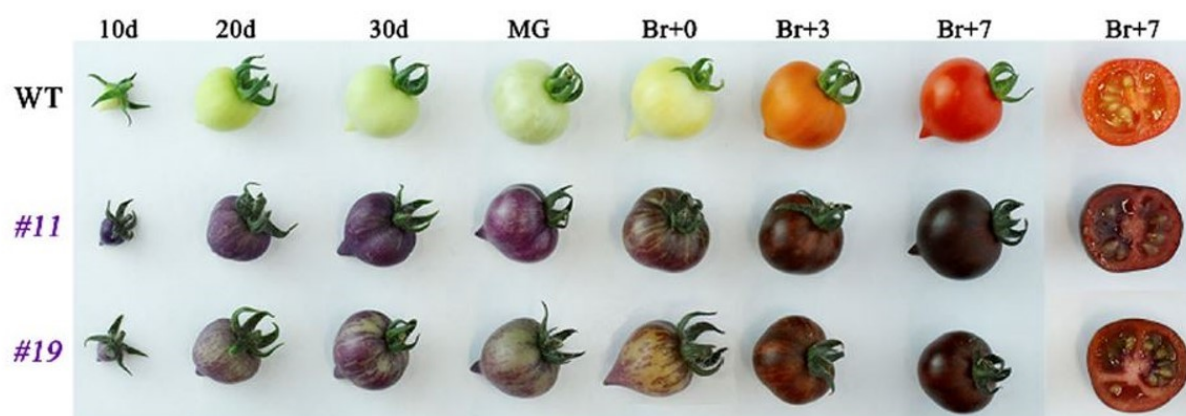


Figure 1. 22 Phenotypic characteristics of WT and SIMYB75 fruits at different developmental stages, ©Jian et al. 2019.

1.3.5 The *PhAN4* gene and the generation of transgenic tomato HRCs and plants thereof

Studies have demonstrated that pathway engineering approaches implying the sole use of MYB factors belonging to the SG6 clade are sufficient to restore the biosynthesis of anthocyanins by promotion of the transcription of their bHLH partners and, therefore, by reconstituting the MBWW (Zhang et al., 2019). As an example, the tomato *ANT1* gene encodes a MYB transcription factor belonging to the SG6 clade, highly homologous to the *Antirrhinum Rosea*. It has been demonstrated that ectopic expression of *ANT1* from a tomato wild relative (*S. chilense*), induces purple spotting on the epidermis of tomatoes (Mathews et al., 2003; Schreiber et al., 2012). The 35S promoter-driven expression of either the *Solanum lycopersicum* *ANT1* or *AN2* (another SG6 MYB), has been shown to induce anthocyanins production in the flesh and peel of the fruit and in different organs of tomato plants (Kiferle et al., 2015). As already introduced (Jian et al., 2019), upon overexpression of *SLAN2*, together with anthocyanins accumulation in fruits, flower organs and vegetative parts, and an enhancement of the emission of volatile molecules contributing to the aroma of fruits was found, as well (Jian et al., 2019). In addition, *SLAN2* has been related to the variation of levels of specialized metabolites other than anthocyanins, and of fruit softening (Meng et al., 2015). These findings seem to confirm that MYB transcription factors of the SG6 clade can regulate various, sometimes unrelated, processes in tomato, in addition to anthocyanins biosynthesis (Stracke et al., 2001; Zimmermann et al.,

2004). Therefore, an SG6 MYB-based approach may be considered suitable to affect multiple pathways in tomato.

The *Anthocyanin4* gene of *Petunia hybrida* (*PhAN4*) is an SG6 member of a small family of genes encoding very similar MYBs phylogenetically related to the snapdragon *Antirrhinum Rosea*, the tomato *SLANT1*, and other anthocyanin-regulating MYBs from a multitude of plant species involved in the induction of anthocyanins accumulation in different plant parts and in response to different stimuli (Povero, 2011). Since mutation of *PhAN4* eliminates expression of *AN1*, *PhAN4* is considered an upstream regulator of the latter (Spelt et al., 2000). Moreover, the AN4 MYB may contain or control an *AN2-like* gene (Spelt et al., 2000).

A single-gene approach to impact different traits would be greatly desirable in agrospace applications, where several aspects of plant physiology can be affected, influencing growth. For this reason, tomato hairy root cultures and whole MicroTom plants have been obtained in our laboratory by *PhAN4* transgenesis, to possibly devise testbeds and plant ideotypes, respectively, for agro-space application of tomato able to tackle different aspects of plants and human life in space, with the particular goal to counteract of the effects of pro-oxidant chemical species on plants and humans and to gain resistance of plants to other abiotic and biotic stresses related to space cultivation life in containment (Massa et al., 2022).

R. rhizogenes-mediated transformation of plant tissues can lead to the generation of root organ cultures endowed with indefinite growth, high genetic stability and capability to synthesize several either endogenous or exogenous (if genetic engineering is applied) valuable compounds, defined as hairy root cultures (HRCs). The HRC system has allowed the successful production of different specialized metabolites with pharmacological, cosmetic and nutraceutical value. More importantly, HRCs have also been used to elucidate physiological processes and biosynthetic pathways, as well (Gutierrez-Valdes et al., 2020). The HRC system, has, therefore, served as a handier and faster biotechnology tool, before whole plant transformation, to gain initial biological insights into a gene of interest function, spatial and temporal gene expression studies and signaling pathways in plant cell response to a changing environment in several plant species (Ron et al., 2014).

2. AIM OF THE WORK

Novel methods for producing edible fresh plant biomass are expected to provide solutions for long-term crew survival in future deep-space manned missions. Due to cosmic radiation, pro-oxidant stimuli are considered the most prominent for plants artificially adapted to space agriculture. In addition to plant survival, the issue of counteracting overproduction of free radicals generated by harmful ionizing radiation is crucial for human survival in space outposts, as well, and opens the way to the ideal ‘anti-oxidant space fresh food’. Together with the IR and pro-oxidant stimuli, plants intended for space agriculture will have to cope with a plethora of abiotic ‘micro-stresses’ related to the different cultivation environments/systems that will be adopted. Furthermore, plants will have to share with humans future crewed space habitats exposing plants to contaminations possibly leading to diseases and spoiling of fruits.

Despite plants’ intrinsic potential for adaptation to drastic environmental changes (e.g., radiation, atmospheric pressure, gravity etc.), the engineering of plant genotypes able to satisfyingly cope with non-terrestrial conditions is being considered as an approach to further overcome possible hindrances to crop cultivation in space environments. This goal may be achieved by optimization of inherent biochemical and physiological patterns related to space stresses. As a main example, specific and targeted regulation of anti-oxidant specialized metabolites biosynthesis can give the plant itself protection against several environmental (abiotic and biotic) challenges.

This PhD work has been part of the ‘BIOExTREME’ and ‘HORTSPACE’ projects (funded by the Italian Space Agency) carried on by the Biotechnology Laboratory of ENEA and represents a preliminary study in the development of crop plant ideotypes with future applications in the agrospace field. In particular, our efforts were focused on the agrospace-oriented improvement of MicroTom, a tomato cultivar considered suitable to these applications thanks to its small size, and availability of data in simulated extraterrestrial conditions.

In this work, MicroTom cells have been engineered through the ectopic expression of the anthocyanin R2R3-MYB transcription factor *AN4* from *Petunia hybrida* (*PhAN4*), to possibly achieve multiple favourable effects on MicroTom traits, as indicated by literature for homologous R2R3-MYB transcription factor belonging to the SG6 clade.

To elucidate the *PhAN4* gene function in tomato, a simplified MicroTom-derived HRCs system was firstly established. Transcriptome-wide RNA-seq and mass spectroscopy analysis were used to evaluate the *PhAN4*-related modulation of expression of genes involved in the anthocyanin biosynthetic pathway and the possible creation of novel expression patterns for genes involved

in other metabolic pathways (i.e. reactive oxygen species metabolism, abiotic and biotic stress stimuli).

Data obtained from the study on AN4-engineered MicroTom HRCs opened up to the whole MicroTom plant engineering. AN4 T₃ generation plants were then characterized at both genetic (using the Targeted Locus Amplification technique) and phenotypic (through the measurements of the most common biometric and fluorimetric parameters) levels. Additional research was carried on to evaluate the potential of *PhAN4*-biofortification to protect humans (as the basis of anti-oxidant space food) and plants themselves from oxidative damage. To evaluate if engineered ripe fruits have differential capacity to counteract the generation of ROS compared to wild type, ESR analyses were conducted on fruits exposed to high dose gamma rays. Moreover, we investigated if the *PhAN4*-based engineering preserve plant protein oxidation and folding. Indeed, for proteins to be functional within a cell requires coordinated folding processes to obtain a correct 3D shape. Disruptions to protein folding, that can occur in space due to ionizing radiation, can have profound biological implications for all organisms, including plant cells, leading to dysfunctions.

To study how AN4 MicroTom plants perform upon ionizing radiation, we exposed seeds and, for the first time, plants at two different developmental stages (30 and 45 DAS), to gamma rays at doses that plants could possibly experience during a long-term space mission in different hypothesized scenarios. In addition, we evaluated the effects of irradiation at one generation distance in time on offspring obtained from plants irradiated at 30 DAS. Plant responses to gamma rays were evaluated following two approaches: biometric parameters were monitored to detect changes that occurred at both phenotypic and phenological levels, while multiparametric fluorescence-based indices were used to indirectly measure changes that occurred at a physiological level in a non-destructive manner.

The information obtained will be useful in developing appropriate ideotypes for future long-term space exploration.

3. MATERIALS AND METHODS

3.1 Plant material

Solanum lycopersicum L. miniature-dwarf-determinate cv. MicroTom (Scott and Harbaugh, 1989) was used as the starting and control plant material for the AN4 HRCs-based system and transgenic plants. Small size (i.e., approximately 15 cm height at maturity and a volume of $\sim 0.0156 \text{ m}^3$), short life cycle (i.e., 90-100 days from sowing to fruit ripening), high productivity (i.e., 20-30 fruits/plant; 2-4 g/fruit) and capacity to grow under fluorescent or LED lights and hydroponic conditions, make MicroTom ideal for optimizing space and seed-to-seed time for agrospace application of tomato plants.

The AN4-based HRCs expression system, served as an initial, handier and faster biotechnology tool, to gain biological insights in *PhAN4* gene function in transformed tomato cells, before accomplishing it in whole *PhAN4* MicroTom engineered plants.

3.2 Hairy Root Cultures generation

S. lycopersicum (cv. MicroTom) clonal hairy root lines were obtained from wild type leaf explants by infection with *R. rhizogenes* A4 (ATCC, 43057TM) harboring either the 35S:*PhAN4* or no additional construct, as described in Rodriguez-Hernandez et al., 2020. Fresh growing hairy roots were obtained after 8–10 days. *R. rhizogenes* was eliminated with decreasing Cef concentrations (0.25, 0.125, and 0.05 $\mu\text{g/ml}$) until no antibiotic was added. Hairy root biomass harvested for analysis was carefully handled, pulverized in liquid nitrogen, and immediately stored at -80°C . For metabolite content and anti-oxidant properties analysis, HRCs were lyophilized in a freeze-dry system (FreeZone Labconco, Kansas City, MO, USA).

Standard PCR assays were performed on genomic DNA of kanamycin-resistant hairy root clones (extracted with NucleoSpin Plant II Kit; Macherey-Nagel; Duren, Germany) with primers specific for *PhAN4*, *rol B*, *rol C*, *virC1*, respectively (Table 3.1), to select hairy root lines carrying *PhAN4* transgene clean from *R. rhizogenes* in the tissue culture.

Table 3.1 Primer identification (ID) as described in materials and methods for both HRCs and plants PCR screening (for: forward, rev: reverse).

PCR Primer ID	Sequence 5'-3'	Amplified sequence length
RolB for	ATGGATCCCAAATTGCTATTCCTTCCACGA	776 bp
RolB rev	TTAGGCTTCTT CTTCAGGTTTACTGCAGC	
RolC for	TGTGACAACAGCGATGAGC	487 bp

RolC rev	GATTGCAAACCTTGCACTCGC	
VirC1 for	AATGCGTCTCTCTCGTGCAT	425 bp
VirC1 rev	AAACCGACCACTAACGCGAT	
PhAN4for	ATGAAAACCTTCTGTTTTTACGTCGTC	768 bp
PhAN4rev	TTATAGTAATTCCCAGAGGTCAGCATC	
AN4_FW	GGGGACAAGTTTGTACAAAAAAGCAGGCTGGATCCGAAA AAAATGGGTA	
AN4_RV	GGGGACCACTTTGTACAAAGAAAGCTGGGTTTCAGTGCAGT TCGTTCAACAA	
qPCR primer ID		
SIAct41for	GCTCTTGACTATGAACAGGAAC	126 bp
SIAct41 rev	AAGGACCTCAGGACACCG	
q PhAN4 for	GCCGAAGTCGTGTCCAGAGACG	105 bp
q PhAN4 rev	AAATGCTGAAGTGCTTTCAGTTG	

3.2.1 Effects of *PhAN4* on tomato transcriptome evaluated in MicroTom HRCs

Total RNA from wild type and AN4 HRCs samples was extracted using the RNeasy Plant Mini Kit (Qiagen; Valencia, CA, USA). RNA was quantified using the 4TM Fluorometer (ThermoFisher Scientific, Waltham, MA, USA) and assayed through Agilent 2100 Bioanalyzer for quality and Integrity Number (RIN) evaluation. Samples with $8 \leq \text{RIN} \leq 10$ were considered. For each sample, equal amounts of RNA (2µg) extracted from three biological replicates were pooled for cDNA library construction. First, the poly-A mRNA in the total RNA was pulled down using poly-T oligo-attached magnetic beads. After purification, the mRNA was fragmented at 95 °C for 2 min along with RT primer and first-strand buffer. The fragmented RNA was used for the synthesis of the first-strand cDNA by adding DTT, dNTPs, RNase Inhibitor and SMARTScribe. All chemicals were incubated for 2 hours at 42 °C. This was followed by second-strand cDNA synthesis using template-switching oligonucleotide and an additional 1 µL of SMARTScribe. Subsequently, the cDNAs were purified using two rounds of AMPure beads. The samples were enriched by using PCR to create the final library. A final fragment size purification step was performed using the Blue Pippin system selecting fragments between 250-500 bp. The libraries were confirmed by electrophoresis on a 1% TAE agarose gel and the Agilent BioAnalyzer 2100® after purification by AMPure bead. The average read size was estimated at 250–350 bp. The libraries were sequenced at Novogene Corporation using two lanes of an Illumina HiSeq4000 system with a pair-end run of 2x150 bp.

Raw RNA-seq libraries were analyzed according to the bioinformatic in-house pipeline (https://github.com/KoesGroup/Snakemake_hisat-DESeq). The raw reads (in FASTQ format)

generated from sequencing were cleaned using Trimmomatic v0.36 (Cock et al., 2009) by removing adaptor-polluted reads, reads with unknown sequences “N” accounting for more than 5% and low-quality reads (with a mass value less than 10 and proportion of the total number of bases in the reads greater than 20%). The clean reads were mapped to the *S. lycopersicum* reference genome sequence version ITAG 4.0 (Sato et al., 2012) downloaded using the Sol Genomic Network database (Fernandez-Pozo et al., 2015). Two programs were used for this purpose, Hisat2 v. 2.1.0 (Kim et al., 2015) and STAR v 2.5.2b (Dobin et al., 2013). The mapping results were compared with Picard Tools CollectAlignmentSummaryMetrics v. 1.138 (<http://broadinstitute.github.io/picard/>). The STAR mapping results were selected for further analysis. BAM files were transformed to a subread matrix file using Rsubread v. 1.34.4 R package (Liao et al., 2019). Differential expression analysis was performed with DESeq2 R package v. 1.22.2 (Love et al., 2014). Genes with an adjusted P-value of ≤ 0.001 and a $\log_2$ fold change of ≥ 2 were defined as differentially expressed (Benjamini and Hochberg, 1995). The list of DEGs (Cluster 1 – PhAN4/WT < 1 and Cluster 2 – PhAN4/WT > 1) was analyzed using g:Profiler (Reimand et al., 2007) with the default parameters, using Organism ‘*Solanum lycopersicum*’. No terms were statistically significant under the “Measure underrepresentation” option. The results were exported as .csv and uploaded into R Studio where they were plotted with ggplot2. The gene functional annotation was performed by sequence homology search with different protein data sets using BLASTP and Protein domains search using InterPro Scan.

3.2.2 Phenylpropanoids identification

Anthocyanin profile was carried out on representative AN4 HRCs by liquid chromatography coupled to high-resolution mass spectrometry (LC-HRMS) as reported before (Diretto et al., 2019) with slight modifications. Briefly, 3 mg (dried weight) of ground hairy roots were re-suspended in 600 μ l of 85:15 MeOH: 1N HCl, vortexed, shaken in Mixer Mill (MM) for 15’ at 20 Hz frequency and gently mixed at 4°C O.N. Samples were then centrifuged at 20,000 g for 20 min, the supernatant recovered, completely dried and re-suspended in 600 μ l of spiked (with 0.5 μ g/ml formononetin, as internal standard) 75% MeOH + 0.1% formic acid. Samples were then centrifuged 10 minutes at 20,000 g at RT, and the supernatant was transferred to HPLC vials for MS analysis with a Q-Exactive mass spectrometer (Thermo Fisher Scientific), coupled to an HPLC system equipped with a photodiode array detector (Dionex). LC separation of anthocyanins was performed injecting 5 μ l of sample on a C18 Luna reverse-phase column (100 x 2.1 mm, 2.5 μ m; Phenomenex), using as mobile phase water + 0.1% formic acid (A) and acetonitrile + 0.1% formic acid (B) at a total flow rate of 250 μ l/min. The separation was

developed using 5% B for 0.5 min, followed by a 24 min linear gradient to 75% B. The ionization was performed using heated electrospray ionization (HESI) source, with nitrogen used as sheath and auxiliary gas, set to 35 and 10 units, respectively. The vaporizer temperature was 250 °C, the capillary temperature was 30 °C, the spray voltage was set to 3.5 kV, the probe heater temperature was 390°C and the S-lens RF level was set at 50. The acquisition was performed in the mass range 110-/1600 m/z both in positive and in negative ion mode with the following parameters: resolution 70,000, microscan 1, AGC target 1e6, maximum injection time 50. UV-VIS detection was continuous from 220 to 700 nm. All solvents used were LC-MS grade (Merck Millipore). Identification was achieved based on accurate masses and by comparison with authentic reference substances. The ion peak areas were normalized to the ion peak area of the internal standard (formononetin).

3.2.3 Determination of total phenolics accumulation level and DPPH assay

Total phenolic content of AN4 MicroTom HRC was estimated by colorimetric assay with modified (Inglett et al., 2010) Folin–Ciocalteu reagent (Şensoy et al., 2006). Briefly, 4.25 ml of de-ionized water was mixed with 0.25 ml of ethanolic extract diluted 1:5 with 80% (v/v) ethanol and 0.25 ml of Folin-Ciocalteu reagent. After seven minute-incubation in the dark at room temperature, 0.5 ml of saturated sodium carbonate solution (20%) was added, and the mixture was incubated for 40 minutes in the dark at room temperature. Absorbance was measured at 725 nm using an UV-VIS spectrophotometer. A standard curve was prepared with gallic acid, as reference standard. Final values were obtained by interpolating the absorbance values recorded for tomato hairy root extracts with gallic acid calibration curve. The total phenolic content was expressed as µg of gallic acid equivalents (GAE) / g of dry weight. Each analysis consisted of triplicate measurements of each sample and data were averaged over the three measurements. Phenolic compounds accumulating in AN4 HRCs were extracted (Morishita et al., 2007) to apply the DPPH free radical scavenging method in order to establish their anti-oxidant properties. Ground, freeze-dried tomato HRC (30 mg) were extracted with 0.6 ml EtOH 80% (v/v), shaken in a water bath at 80 °C for 40 min and then centrifuged at 3,000 rpm for 15 min. The recovered supernatant was filtered through polytetrafluoroethylene membrane (0.45 µm) and stored at –20 °C. The antioxidant capacity of the ethanolic extracts was spectrophotometrically tested by DPPH (1,1-diphenyl-2-picrylhydrazyl) (Li et al., 2010), with some modifications. Briefly 0.25 ml of diluted ethanolic extract was added to 2.9 ml of 0.06 mM DPPH working solution. The mixture was shaken and allowed to stand at room temperature, in the dark, for 30 min. Absorbance was measured at 515 nm using an UV-VIS spectrophotometer. Lower absorbance

values of reaction mixture indicate higher free radical scavenging activity. The inhibition of free radical DPPH was expressed as DPPH scavenging effect (% inhibition) $I\% = \{(A_0 - A_1)/A_0\} \times 100\}$, where A_1 and A_0 are the absorbance values of blank and of tested samples, respectively. Trolox (vitamin E equivalent antioxidant) was used as reference standard. Each analysis consisted of triplicate measurements of each sample and data were averaged over the three measurements.

3.2.4 Electron Spin Resonance (ESR) Spectroscopy before and after gamma irradiation

The molecular species accumulating upon the ectopic expression of *PhAN4* were investigated for possible efficient maintenance of the ability to counteract the generation of reactive oxygen species upon strong ROS inducers such as ionizing radiations. Gamma rays were used to generate peroxy radicals (which are proportional to the number of paramagnetic species present in the samples) in lyophilized HRC powder. We explored ROS formation before and after 2 kGy absorbed dose in AN4-1 and control HRCs sample sets by ESR Spectroscopy measurements. Not irradiated sample sets were used as reference. Each set consisted of two replicates. For each ESR analysis, 8 ± 0.1 mg of HRC lyophilized powder was split in two PT-Capillaries (NOX-A.8.1-PT NOXYGEN, Holland) that were then inserted in a conventional quartz sample tube (o.d./i.d. of 4/3 mm) closed by a plastic lid. Irradiated samples were analyzed straight after the end of irradiation and ESR signals and were normalized to the sample mass. ESR measurements were acquired using an ESR e-scan spectrometer (Bruker) operating in the X-band frequency (9.4 GHz) with a field modulation frequency of 86 kHz and modulation amplitude of 5.152 G. The ESR spectra were recorded at a central magnetic field of 3466 Gauss with a sweep width of 160 G, microwave power of 0.14 mW, microwave frequency of 9.75 GHz. The ESR spectra reported in this work derived from the accumulation of four scans. Bruker WinEPR data processing software was used for data elaboration.

3.2.5 UV-VIS absorbance analysis before and after gamma irradiation

UV-VIS spectra were obtained from crude extracts of AN4-1 and control HRCs. Briefly, lyophilized HRCs were ground in liquid nitrogen and the resulting powder was finely homogenized using an Ultraturrax homogenizer (IKA) in water:HCl (100:1, v/v). Samples were incubated at 500 rpm for 1 hour at R.T., clarified by centrifugation at 11,000 g for 30 minutes. The resulting supernatants were examined at 280–600 nm, by a UV-spectrophotometer (Lambda 950, Perkin Elmer, USA) at R.T. with a slit width of 2 nm, using a 10 mm cell.

3.2.6 Photoluminescence analysis before and after gamma irradiation

Samples were finely ground in liquid nitrogen with mortar and pestle, resuspended and homogenized in phosphate-buffered saline pH 7.2 (PBS, 1:3 w/v) containing a protease inhibitor cocktail (Complete, Roche) to extract soluble proteins. Photoluminescence emission spectra of extracts were determined before and straight after 2 kGy absorbed dose in AN4-1 and in control HRCs dried biomass sample sets. Two replicates per set were poured into quartz cuvettes with an optical path length of 1 cm (104F-QS, Hellma, Germany). The emission spectra were recorded using the Edinburgh Instruments FS 5 spectrometer in the range 300-800 nm with 280 nm excitation wavelength. The recorded spectra were mass-normalized and corrected for background scattering (reference: extraction buffer).

3.3 AN4 MicroTom plants generation

Engineered AN4 plants have been generated at the Department of Plant Development and (Epi) Genetics, Swammerdam Institute for Life Sciences, University of Amsterdam (Amsterdam, The Netherlands) by the R. Koes group. Briefly, *Anthocyanin4* (*PhAN4*) cDNA (GenBank: HQ428105.1) from petals of *Petunia hybrida* cultivar Violet 30 was amplified with primers containing attB-1 and -2 recombination sites (highlighted below by underlining):

AN4_FW:

GGGGACAAGTTTGTACAAAAAAGCAGGCTGGATCCGAAAAAAATGGGTA:

AN4_RV:

GGGGACCACTTTGTACAAGAAAGCTGGGTTCAGTGCAGTTTCGTTCAACAA.

BP recombination of PCR product and pDONR221 P1-P2 (RU Ghent) produced the entry clone. The resulting pEntry construct was recombined with pKGW.0 (RU Ghent; www.psb.ugent.be/gateway/) using LR clonase, obtaining the 35S:AN4 construct. The construct has been introduced into wild-type MicroTom leaf disks by *Agrobacterium tumefaciens*-mediated transformation (as described for *Petunia hybrida* by Kroon, 2004. Transgenic lines were screened for *PhAN4* gene copy number by qRT-PCR using the primers described in Table 1. Wild type MicroTom and the T₃ generation of a homozygous line (AN4-M) and AN4-P₂ hemizygous plants harbouring 1 copy of the *PhAN4* gene per haploid genome were considered for this study. Namely, these two transgenic lines were denoted as ‘AN4-M’ and ‘AN4-P’, due to the magenta (M) and purple (P) colour phenotype of ripe and breaker fruits, respectively.

3.3.1 AN4 MicroTom plants growth conditions

Plants were cultivated in a containment greenhouse biosafety level 2 (BSL2) at the ENEA Casaccia Research Center (Rome) in hydroponic conditions. In particular, plants were cultivated in mesh pots containing a mix of horticultural perlite and vermiculite in a 1:1 ratio with nutrient solution (1 gr/l) (Idrofill base, K-Adriatica, Italy). The nutrient solution's pH was maintained between 5.5 and 6.5 and the electrical conductivity (EC) was set between 2.2 and 2.5 dSm⁻¹ (Groline monitor, Hanna Instruments, Italy). The illumination system consisted of natural light integrated with artificial light (LumiGrow PRO 650 lamps, blue LED 70 $\mu\text{mol m}^{-2} \text{s}^{-1}$, white LED 70 $\mu\text{mol m}^{-2} \text{s}^{-1}$, red LED 160 $\mu\text{mol m}^{-2} \text{s}^{-1}$) (LumiGrow, California, USA), with a photoperiod of 16/8 hours day/night and a temperature of 25/16 °C day/night.

3.3.2 AN4 MicroTom plants genetic characterization by TLA and sequencing

Targeted Locus Amplification (TLA) technology (De Vree et al., 2014) was accomplished to identify genetic alterations in the transgene (i.e., identify single nucleotide variants (SNVs), transgene integration site(s), breakpoint sequences between the transgene and tomato genome, to assess the possible presence of structural variants surrounding the transgene integration site and to have confirmation of zygosity to exclude multiple integration sites in the MicroTom transgenic lines sites selected for further analysis. TLA was conducted on genomic DNA (gDNA) extracted from mature leaves of T₃ generation plants from AN4-M and AN4-P plants that had shown to harbour 1 copy of the *PhAN4* gene per haploid genome. The gDNA was extracted using the NucleoSpin Plant II kit (Macherey-Nagel; Duren, Germany), following the user manual. The gDNA was further quantified using the Qubit 4TM Fluorometer (ThermoFisher Scientific, Waltham, MA, USA). Samples were processed according to Cergentis TLA protocol (Cergentis, B.V., Utrecht, The Netherlands). Two primer sets were designed on the transgene (Table 3.2) and were used in individual TLA amplification.

Table 3.2 Primers used in individual Targeted Locus Amplification (TLA).

Primer set	Name/View Point	Direction	Binding position	Sequence
1	T356	RV	49	TTGTAGAGAGAGACTGGTGA
		FW	131	GGTTCTTATAGGGTTTCGCT
2	NEO	RV	7548	TGTTCCGCCAGGCTCAAGG
		FW	7685	GTAGCCGGATCAAGCGTATG

PCR products were purified and the Illumina Nextera flex protocol was used to prepare the libraries subjected to the Illumina sequencing (Illumina Inc., San Diego, California). The

obtained reads were mapped using BWA-SW version 0.7.15-r1140 with the settings `bwasw -b 7` as described in Li and Durbin, 2010. The NGS reads were then aligned to the transgene (TG) and the host genome. The tomato GCF_000188115.4_SL3.0 was used as a host reference genome sequence. The presence of single nucleotide variants (SNVs) was determined using samtools mpileup version 1.3.1 (Li, 2011). SNVs reported met the following criteria: allele frequency (the relative amount of reads with the variant, compared to total coverage on the variant position), the variant is in the data of both primer sets, for at least one of the primer sets the coverage is $\geq 30X$, the variant is identified in both forward and reverse aligning sequencing reads, low-frequency variants (between 5-20% mutant allele frequency) are not found with similar frequencies in the negative control. In addition, integration sites are detected based on coverage peak(s) in the genome and on the identification of fusion-reads between the TG sequence and the host genome.

3.3.3 AN4 MicroTom plants phenotypic characterization

MicroTom wild type, AN4-M and AN4-P₂ plants were phenotypically characterized using the following biometric parameters: plant height (measured using a digital calliper), overall leaf area (measured using Image J version 1.8.0, National Institute of Health, USA), number of leaves per plant, number of flowers per plant, number of fruits per plant, fruits weight at the ripe stage, equatorial diameter of fruits (determined by a digital calliper), number of seeds. Furthermore, the percentage of plantlets obtained from seeds was evaluated using the following formula:

$$\text{Percentage of plantlets (PP)} = \left(\frac{\text{number of plantlets after 15 DAS}}{\text{total number of seeds}} \right) \times 100$$

To investigate how the irradiation procedures can influence metabolic processes, non-destructive fluorimetric measurements were conducted on whole irradiated plants and descendants using the Multiplex Research™ (Force-A, Orsay, France) device. Multiplex Research™ is a hand-held multi-parameter optical sensor based on light-emitting-diode excitation and filtered-photodiode detection that is designed to work on leaves, fruits and vegetables (Ghozlen et al., 2010; Diago et al., 2016). Fluorimetric analyses were performed once a week throughout the plant cycle to measure the following indices: SFR, FLAV, ANTH and NBI indices (Ghozlen et al., 2010; Tremblay et al., 2012). The SFR index is linked to the chlorophyll (Chl) concentration of leaves and increases with increasing Chl concentration. It is a fluorescence ratio (SFR) of far-red Chl emission (FRF, 735 nm) divided by red Chl emission (FR, 685 nm) under red (FRF_R and FR_R, respectively) (Buschmann, 2007). The FLAV index, which is proportional to flavonols

concentration, compares the Chl fluorescence intensity emitted as far-red fluorescence under ultraviolet (FRF_UV) and red excitation (FRF_R). The ANTH index is the logarithm to base 10 of the ratio of far-red fluorescence (FRF) excited at two different wavelengths (red FRF_R and green FRF_G) and it is proportional to anthocyanin content. The NBI index represents the ratio between the primary and secondary metabolism (Chl index/FLAV index). A disc with an aperture of 8 cm was used in front of the optical unit to enable illumination and measurement of an area of approximately 13 cm² by maintaining a constant distance of 10 cm between the light source of the device and the measured plant surface. A single measurement consisted of 500 individual excitation flashes and detection of the respective fluorescence signals for a total acquisition time of less than 1s (Agati et al., 2013).

3.3.4 AN4 MicroTom gamma irradiation procedures

As extensively described in the literature (Mousseau and Møller, 2020), several logistical challenges make difficult to set up ground-based chronic exposure experiments. For this reason, the majority of the studies have been conducted using acute irradiation and considering the final absorbed dose. To test how wild type and AN4 plants respond to the irradiation with gamma rays, acute treatments were supplied at the Calliope facility to both dry seeds and plants at two developmental stages (30 and 45 days after sowing, DAS). At the 30 and 45 DAS life stages MicroTom has completed approximately 30% and 50% of its entire life cycle, respectively. In particular, 0.5 Gy (6 Gy/h dose rate), 5 Gy and 30 Gy (60 Gy/h dose rate) ⁶⁰Co gamma radiation absorbed dose were delivered to plants. These doses were chosen based on both estimation and radiation data obtained during the last years from HZETRN, MARIE, GOES-8 and RAD (Hu et al., 2009; Hassler et al., 2014; Guo et al., 2017; Chancellor et al., 2018). Due to the complexity in predicting the precise dose to which plants will be subjected in outer space missions, we hypothesized different scenarios of cultivation trials (Figure 3.1). In these scenarios, we considered the most recent value obtained from RAD on board on the Curiosity Rover at Gale crater for GCRs (0.210 ± 0.040 mGy/day) and the large event occurred in October 1989 for SEPs (1454 mGy/hour) (Hassler et al., 2014; Chancellor et al., 2018). Five plants per radiation dose group were used. Both irradiated plants and not-irradiated controls were subjected to the same

procedures (including the transfer to the irradiation facility), to ensure a complete homogeneity of treatment.

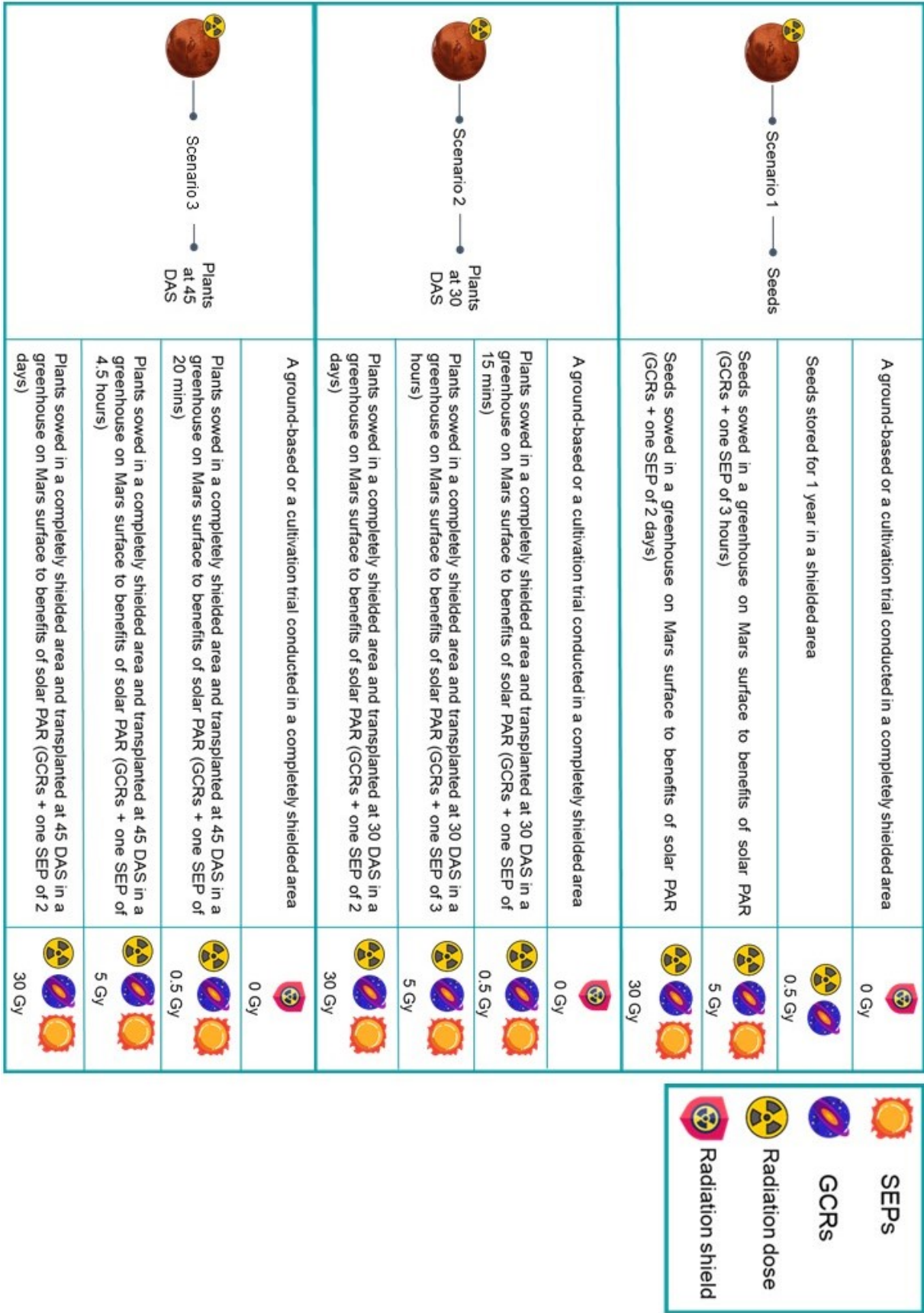


Figure 3.1 Scenario hypothesized for cultivation trials on Mars surface.

3.3.5 Plant response evaluation to gamma irradiation

3.3.5.1 Non-destructive morphometric and fluorimetric analyses

Analysis of plant response to gamma irradiation was performed through non-destructive morphometric and fluorimetric analyses that were initiated 30 days after sowing in the case of plants obtained from irradiated seeds. Plants irradiated at 30 and 45 DAS were subjected to measurements at three time points right after irradiation (10 minutes, 1 hour, 3 hours) and, thereafter, proceeded weekly (days after irradiation, d.a.i.) throughout the plant cycle until fruits set. The parameters follow-up of offspring obtained by self-pollination of plants irradiated at 30 DAS, was conducted at three time points (30, 60 and 90 days after sowing with the latter that represents the time point at which plants were harvested).

Morphometric and fluorimetric analysis were performed as described in section 3.3.3 for the characterization of non-irradiated AN4 MicroTom plants.

3.3.5.2 Non-destructive fluorescent emission measurements

Fluorescent emission measurements were carried out to track the performances of the photosynthetic apparatus and its capacity to recover from the stress that plants have been subjected to. The photosynthetic maximum photochemical efficiency (F_v/F_m) and the maximum fluorescence yield (Y_F) were evaluated as plant stress indices using the Plant Stress Kit (PSK) (Opti-sciences, Hudson, USA). Three fully developed leaves from each replicate were clumped with leaf clips for a 30 min dark adaptation before fluorescence measurements. The maximum photochemical efficiency F_v/F_m was calculated on the basis of the equation: $F_v/F_m = (F_m - F_0) / F_m$, where F_0 = initial fluorescence, F_m = maximal fluorescence, and F_v = variable fluorescence (F_m / F_0) (Baker and Rosenqvist, 2004).

3.3.6 AN4 MicroTom fruits antioxidant features characterization

3.3.6.1 Anthocyanins quantification

Three mg (dried weight) of mature fruits were ground and re-suspended in 600 μ l of 85:15 MeOH: 1N HCl, vortexed, shook in Mixer Mill (MM) for 15' at 20 Hz frequency and gently mixed at 4°C O.N. Samples were then centrifuged at 20,000 g for 20', the supernatant recovered, completely dried and re-suspended in 600 μ l of spiked cold (0.5 μ g/ml formononetin, Sigma-Aldrich) 75% MeOH + 0.1% formic acid. Total anthocyanins content was measured by spectrophotometric analysis (Shimadzu, Duisburg, Germany) following the pH-differential method as described in (Brito et al., 2014) and using the extinction coefficient of the most abundant anthocyanin (petunidin-3-(p-coumaroyl)-rutinoside-5-glucoside).

3.3.6.2 DPPH radical scavenging activity assay, ESR and Photoluminescence emission spectra analysis

Given a potential use of *PhAN4*-biofortified plants for agro-space cultivation and as nutraceuticals to protect humans from oxidative damage, mature fruits were investigated for possible efficient maintenance of the ability to counteract the generation of reactive oxygen species upon potent ROS induction through high dose gamma irradiation. Firstly, we investigated the DPPH radical scavenging activity assay as described in section 3.2.3. Then, gamma rays were used to generate peroxy radicals (which are proportional to the number of paramagnetic species present in the samples) in lyophilized biofortified fruits powder. Both ESR and Photoluminescence emission spectra analysis, as described in section 3.2.4 and 3.2.5, were used to explore ROS formation and to investigate for efficient maintenance of folding upon high oxidative stress.

3.4 Data analysis

GraphPad Prism version 8 (GraphPad Software, San Diego, CA, USA) was used for all graphical and statistical data processing. In particular, data obtained from phenotypic characterization, total anthocyanin content and DPPH assay were subjected to ordinary one-way ANOVA with multiple comparisons.

To determine the association between investigated traits and the gamma ray absorbed dose, the Principal Component Analysis (PCA) was performed. To analyse input data (all necessarily endowed with different units of measure) by PCA, the standardized method was used and the PCs were based on parallel analysis that performs Monte Carlo simulations on 'random' data of equal dimension of the input data, calculating the eigenvalues for all resulting PCs. Eigenvalues of PCs from the input data were then compared to the mean (and confidence interval) of the corresponding PCs from the simulations. The chosen percentile level was 95% and the number of simulations was 1000 with auto random seed option.

To evaluate the effects of the treatments in relation to the three genotypes, to time and to the interaction between genotype and time, data obtained from gamma irradiation (with the exception of number of fruits, weight of fruits, diameter of fruits and number of seeds) were subjected to two-way ANOVA analysis of variance with multiple comparisons. Number, weight and diameter of fruits and number of seeds that were recorded only once at the Red Ripe stage of fruits, when the experiment ended, were coherently subjected to one-way Anova.

Both one-way and two-way ANOVA analysis were subjected to Tukey's post-hoc test as a statistical hypothesis testing. Significant differences were shown as asterisks, where *p-value $\leq$ 0.05, **p-value $\leq$ 0.01 and ***p-value $\leq$ 0.001 were indicated on respective graphs.

4. RESULTS

4.1 Characterization of AN4 hairy roots cultures

Hairy roots cultures (HRCs) harbouring the 35S:*PhAN4* construct were generated as a handier system compared to whole plants to initially screen *PhAN4* gene function in tomato cells (Massa et al., 2022) (Figure 4.1).

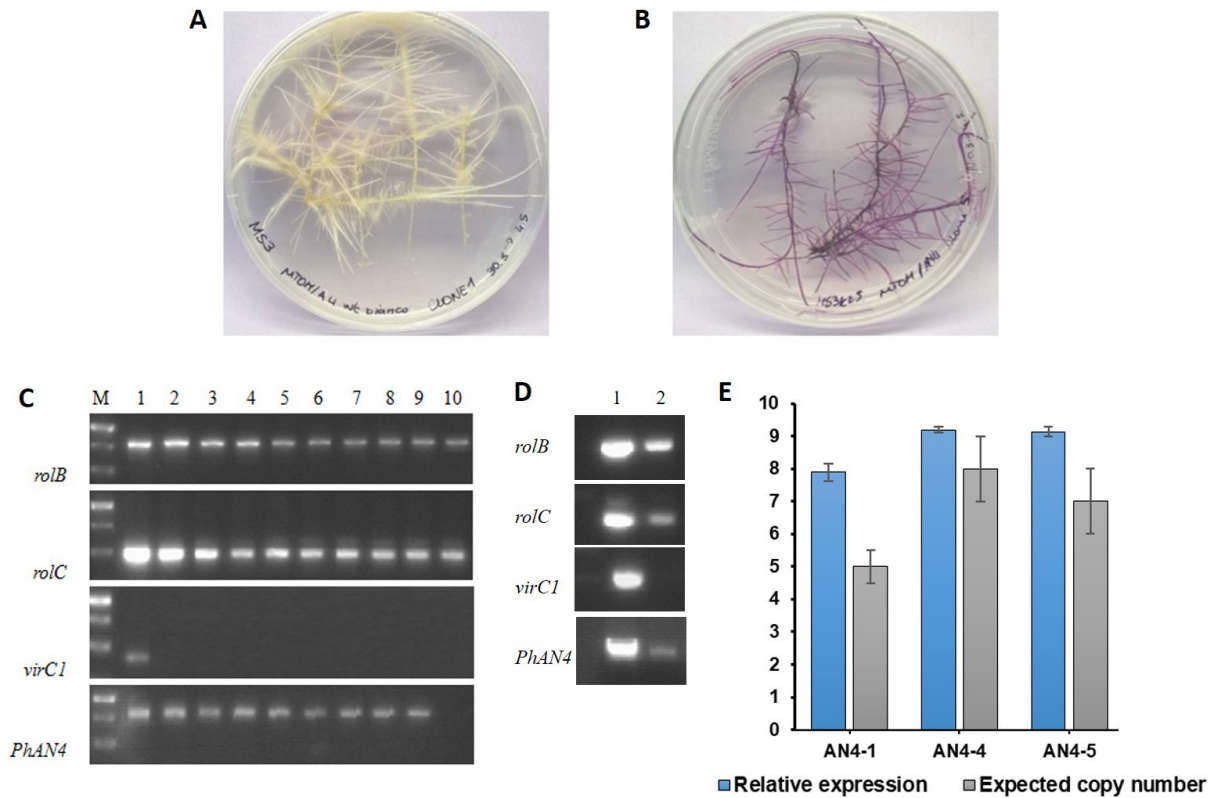


Figure 4. 1 Two representative control (A) and transformed (35S:PhAN4, B) MicroTom hairy roots. PCR analysis on MicroTom HRCs for the detection of specific gene fragments of *rolB*, *rolC*, *virC1* and *PhAN4* from representative, independently isolated HR clones (C). M: DNA marker, Thermo Scientific GeneRuler 1kb DNA Ladder (section 1000 bp - 500 bp); lane 1: positive controls (*A. rhizogenes* for *rolB*, *rolC* and *virC1*, 35S:AN4 plant expression vector for *PhAN4*); lanes 2-9: HRCs obtained with 35S:AN4; lane 10: control HRC. One representative transformed HRC after 1 year-subculture passages. Lane 1: positive controls; lane 2: HRC-extracted genomic DNA (D). Transcript levels and copy number of *PhAN4* in the tissues of 3 independent HRCs (E).

Control HRCs were obtained by transformation with *A. rhizogenes* not containing *PhAN4*. HRCs were collected from independent explants. While control HRCs grew as unpigmented organ cultures (Figure 4.1 A), HRCs generated by 35S:*PhAN4* showed purple pigmentation (Figure 4.1 B) that may vary among clones. Purple pigmentation was present on primary and secondary branches and was maintained on kanamycin selection. PCR screening showed amplification of the expected fragments from genomic DNA (as described in section 3.2), confirming integration

of the necessary root-inducing genes from *A. rhizogenes* and of the *PhAN4* transgene (Figure 4.1 C-D). No transgene loss was observed over time. Control HRCs were negative for *PhAN4* amplification, as expected. Integration of *PhAN4* was estimated at copy numbers ranging from 4 to 8 copies, depending on the selected clone analyzed. *PhAN4* transcript was detected in HRCs harboring 35S:*PhAN4* construct, while they were absent in the control, as expected (Figure 4.1 E).

4.1.1 Transcriptome analysis on AN4 hairy roots cultures

The response to the constitutive expression of *PhAN4* in MicroTom HRCs has been analyzed transcriptome-wide by RNAseq analysis and compared to control HRCs. AN4 HRCs showed a total of 442 differentially expressed genes (DEGs), of which 331 were up-regulated and 111 were down-regulated (Table 4.1 and Appendix I). After Gene Ontology Enrichment (GOE) analysis, 38 up-regulated and 8 down-regulated genes were termed (Appendix II). For up-regulated DEGs, 3 GO categories were assigned: Molecular Function (MF), Biological Process (BP) and Cellular Component (CC) (Figure 4.2).

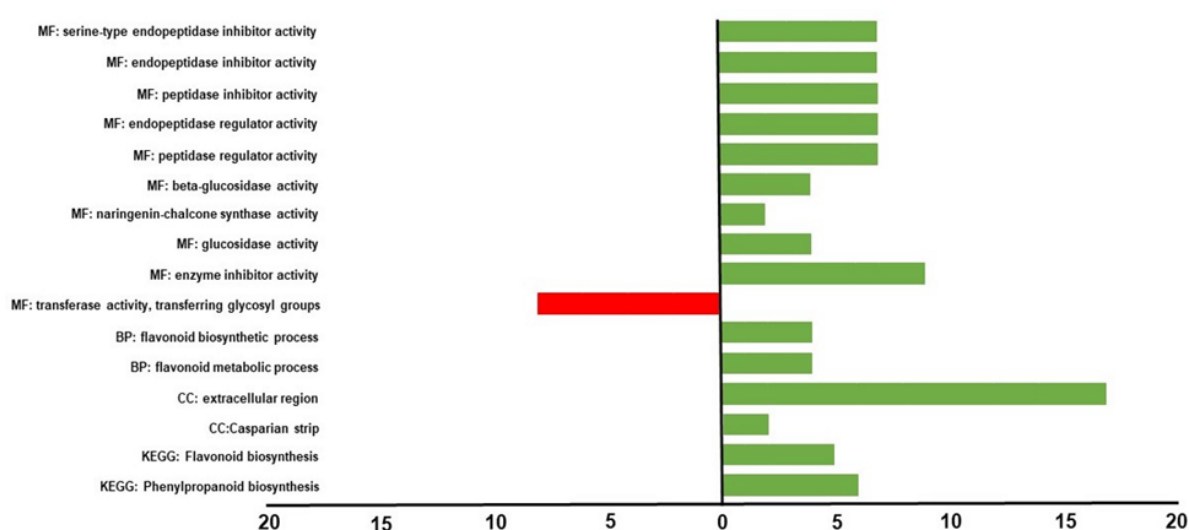


Figure 4. 2 GOs distribution of differentially expressed genes (DEGs). Gene Ontology (GO) terms associated with up-regulated (green bars) and down-regulated (red bars) genes in the PhAN4 HRCs compared to control HRCs, based on “Molecular Functions” (MF), “Biological Process” (BP), “Cellular Component” (CC) and KEGG ontological domain.

Table 4.1 List of the more representative and significant DEGs obtained from the GOE and functional annotation analyses in relation to agrospace application. Function was provided by SolGenomics Network annotation and by specific literature (in bold).

Gene ID	Log ₂ Fc	Gene name	Function	Ref.
Solyc03g020080.3.1	2.017930333	<i>SIP111</i>	Pin-II type proteinase inhibitor / biotic (herbivorous and insects resistance), abiotic (drought/heat)	(Fan et al., 2020)
Solyc03g020030.3.1	10.72904412	<i>SIP116</i>	Pin-II type proteinase inhibitor / biotic (wound stress, insects resistance), abiotic (heat)	(Fan et al., 2020)
Solyc11g020960.2.1	2.362943887	<i>SIP151</i>	Proteinase inhibitor II / biotic (wound stress)	(Fan et al., 2020)
Solyc08g080630.3.1	2.33279266	<i>SIP131</i>	Proteinase inhibitor 1 / biotic (TSWV infection), abiotic (drought)	(Fan et al., 2020)
Solyc10g086090.2.1	2.247200225	<i>SIP147</i>	Trypsin inhibitor 1 / abiotic (drought)	(Fan et al., 2020)
Solyc10g086100.2.1	2.406338004	<i>SIP148</i>	Proteinase inhibitor / abiotic (heat)	(Fan et al., 2020)
Solyc01g059965.1.1	3.476158885	<i>SIGluB</i>	Beta-1,3-glucanase / biotic (defence against pathogens: <i>C.fulvum</i>, <i>P.infestans</i>)	(van Kan et al., 1992; Fan et al., 2021)
Solyc09g091510.3.1	12.25514456	<i>CHS</i>	Chalcone synthase 1 / phenylpropanoids biosynthesis	(Zhang et al., 2019)
Solyc05g053550.3.1	9.83030415	<i>CHS</i>	Chalcone synthase 2 / phenylpropanoids biosynthesis	(Kalt et al., 2020)
Solyc02g083860.3.1	6.299607501	<i>F3H</i>	Flavonoid-3-hydroxylase / phenylpropanoids biosynthesis	(Aoki et al., 2010; Zhang et al., 2019)
Solyc02g085020.3.1	10.09460014	<i>DFR</i>	Dihydroflavonol reductase / phenylpropanoids biosynthesis	(Bongue-Bartelsman et al., 1994)
Solyc01g106650.3.1	3.003680242	-	Xyloglucan endotransglucosylase / cell elongation	(Li et al., 2020a)
Solyc11g011210.2.1	2.189943987	<i>RSI-1</i>	RSI-1 precursor / lateral root initiation	(Taylor and Scheuring, 1994)
Solyc03g093390.3.1	2.390710296	<i>LeEXPB2</i>	Expansin-B15-like / sexual reproduction	(Sundaresan et al., 2016)
Solyc08g077910.3.1	3.227703527	-	Expansin-like B1 / sexual reproduction	(Nveawiah-Yoho et al., 2013)

Solyc05g052245.1.1	2.186779111	-	Expansin A8-like / cell wall organization	-
Solyc09g010860.3.1	2.630126243	<i>EXPA4</i>	Expansin 4-like / cell wall organization	-
Solyc02g062510.3.1	2.392338587	-	Peroxidase 72-like / phenylpropanoids biosynthesis	(Nveawia h-Yoho et al., 2013)
Solyc04g080760.3.1	2.42913063	-	Peroxidase 9 / abiotic stress (hypoxia tolerance)	(Andolfo et al., 2014)
Solyc02g077300.2.1	7.236518572	-	Peroxidase 19 / phenylpropanoids biosynthesis, biotic (ToMV infection)	(Andolfo et al., 2014; Safavi-Rizi et al., 2020)
Solyc06g054320.1.1	2.690758759	-	Dirigent protein / lignin biosynthesis	(Paniagua et al., 2017)
Solyc10g055190.1.1	5.216357243	-	Dirigent protein / lignin biosynthesis	(Paniagua et al., 2017)
Solyc10g055200.1.1	2.06850204	-	Dirigent protein / lignin biosynthesis	(Paniagua et al., 2017)
Solyc04g010270.1.1	2.350516375	-	Dirigent protein / lignin biosynthesis	(Paniagua et al., 2017)
Solyc02g076710.3.1	2.307987898	<i>CathB</i>	Cathepsin B-like cystein / biotic (hypersensitive response)	(McLellan et al., 2009)
Solyc05g053890.2.1	-2.032569214	-	UDP-GT-like / flavonoids glycosilation	(Fernandez-Moreno et al., 2016)
Solyc01g096560.2.1	4.109312617	<i>TomLOXD</i>	Subtilisin-like protease / biotic (wound stress, resistance to insects and necrotrophic pathogens)	(Yan et al., 2013)
Solyc07g054840.3.1	3.341226163	AtMYB41	Transcription factor 41 / abiotic (salt tolerance)	(Hoang et al., 2012)
Solyc03g095810.3.1	6.923791313	-	Trichome birefringence-like / xylan acetylation (resistance against microorganisms, cold and drought)	(Zhang et al., 2020)
Solyc10g007970.2.1	6.961895177	-	Transcription factor 77 / abiotic (water deficit)	(Asins et al., 2021)

Solyc07g043690.2.1	2.800732902	<i>SINPR1</i>	3-hydroxyisobutyryl-CoA hydrolase / abiotic (drought stress)	(Li et al., 2019a)
Solyc03g096460.3.1	2.302142017	-	Wound signaling	(Scranton et al., 2013)
Solyc02g080790.3.1	3.317661852	<i>SIDHS</i>	Deoxypusine synthase / osmotic stress and chilling injury	(Wang et al., 2001; Gupta et al., 2013)
Solyc10g081300.1.1	2.564715	<i>SIMC8</i>	Metacaspase 9 / biotic (apoptosis induction by pests), abiotic (drought, cold and salt)	(Liu et al., 2016)
Solyc10g080690.2.1	2.304387313	-	Patatin / abiotic (flooding)	(de Ollas et al., 2021)
Solyc06g073760.3.1	4.071580028	<i>BGL2</i>	Beta-glucosidase / sugar/organic acid ratio tomato fruits / resistance against different pathogens / softening	(Liu et al., 2016)
Solyc12g035225.1.1	9.263765052	<i>RICESLEEPER1</i>	BED zinc-finger / abiotic (salt tolerance)	(Kashyap et al., 2020)
Solyc12g010500.2.1	5.358159809	-	E3 ubiquitin protein / biotic (resistance to <i>X. perforans</i>)	(Shi and Panthee, 2020)
Solyc12g010670.1.1	5.364030417	-	E3 ubiquitin protein / biotic (resistance to <i>X. perforans</i>)	(Shi and Panthee, 2020)
Solyc12g009630.2.1	5.183567126	<i>SICaM3</i>	Calcium-binding protein / biotic (<i>B. cinerea</i>), abiotic (mechanical wounding, salt and cold stress)	(Peng et al., 2014; Shi and Du, 2020)
Solyc02g067750.3.1	6.915338794	<i>CAI</i>	Carbonic anydrase / abiotic (drought stress)	(Li et al., 2020b)
Solyc08g075705.1.1	4.258074963	<i>pTRXy2</i>	Thioredoxin Y2 / ROS detoxification, redox signaling network regulation	(Serrato et al., 2013)
Solyc09g007190.3.1	-2.218315202	<i>PRXL2A</i>	Peroxyredoxina-like 2A / redox regulatory protein	-
Solyc03g098760.2.1	-5.109469505	-	I3 Kunitz-type trypsin inhibitor / biotic (infection <i>Tetranychus</i> species infection)	(Islam et al., 2015; Schimmel et al., 2018)
Solyc09g089490.3.1	-5.211378167	<i>SPI40</i>	Proteinase inhibitor / abiotic (drought, salt) biotic (<i>B. cinerea</i>, TSWV infection)	(Fan et al., 2020)

Solyc06g008760.1.1	-3.056115855	-	Glutharedoxin-C13-like / upregulated in tomato <i>rin</i> mutants	(Kumar et al., 2016)
Solyc07g055610.2.1	-2.055605009	<i>PR1</i>	Resistance protein R1 / biotic (resistance to late blight and <i>F. oxysporum</i> infection)	(Bournival et al., 1989; Pan et al., 2000)
Solyc01g010480.3.1	-2.123574853	<i>KAT1</i>	K ⁺ channel KAT1 / abiotic (tolerance to potassium deficiency)	(Zhao et al., 2018)
Solyc01g102610.3.1	-3.607916378	<i>FRO6</i>	Ferric reduction oxidase 6 / biotic (<i>P. solanii</i> infection)	(Buoso et al., 2019)
Solyc05g010320.3.1	4.308610241	<i>CHI</i>	Chalcone isomerase / phenylpropanoids biosynthesis	(Morita et al., 2014; Li et al., 2019b)
Solyc05g052240.3.1	8.452278685	<i>CHI</i>	Chalcone isomerase / phenylpropanoids biosynthesis	(Ron et al., 2014; Hou et al., 2017)
Solyc11g066580.2.1	16.35234434	<i>F3'5'H</i>	Flavonoid-3',5'-hydroxylase / phenylpropanoids biosynthesis	(Shi and Panthee, 2020)
Solyc08g080040.3.1	8.861102345	<i>ANS</i>	Anthocyanin synthase / phenylpropanoids biosynthesis	(Shi and Panthee, 2020)
Solyc04g078140.3.1	8.35041607	<i>DilFl</i>	Cytochrome B5 / phenylpropanoids biosynthesis (essential for full activity of F3'5'H)	(de Vetten et al., 1999)
Solyc09g082660.3.1	15.05027118	<i>OMT</i>	caffeoyl-CoA-O-methyltransferase / phenylpropanoids biosynthesis	(Roldan et al., 2014; Shi and Panthee, 2020)
Solyc02g062975.1.1	7.081891304	<i>3UGT</i>	UDP-glucose flavonoid 3-O-glucosyl transferases / phenylpropanoids biosynthesis	(Hu et al., 2011; Tohge et al., 2020)
Solyc10g083440.1.1	9.972475187	<i>3UGT</i>	UDP-glucose flavonoid 3-O-glucosyl transferases / phenylpropanoids biosynthesis	(Hu et al., 2011; Tohge et al., 2017)
Solyc12g098590.2.1	16.55105848	<i>3UGT</i>	UDP-glucose flavonoid 3-O-glucosyl transferases / phenylpropanoids biosynthesis	(Hu et al., 2011; Tohge et al., 2020)

Solyc09g059170.2.1	8.326262972	<i>3RT</i>	Anthocyanidin-3-O-glucoside rhamnosyltransferase / phenylpropanoids biosynthesis	(Tohge et al., 2020)
Solyc12g088170.2.1	13.21347105	<i>AAT</i>	Flavonoid-3-O-rutinoside-4'''-O-phenylacyltransferase / phenylpropanoids biosynthesis	(Florio et al., 2021)
Solyc03g025190.4.1	16.80814437	<i>MTP77</i>	Transparent testa 2-like / toxic compound extrusion, regulation of cell turgescence	(dos Santos et al., 2017)
Solyc10g006120.2.1	7.357873128	<i>LDOX</i>	Leucoanthocyanidin dioxygenase / phenylpropanoids biosynthesis	(Pelletier et al., 1997)
Solyc07g052490.3.1	7.173073383	<i>Atv</i>	Myb-like transcription factor Atv / anthocyanin biosynthesis	(Cao et al., 2017)
Solyc10g086290.2.1	8.651275639	<i>SIAN2</i>	AN2-like transcription factor / anthocyanin biosynthesis	(Sun et al., 2019)
Solyc12g005800.2.1	7.517786731	<i>WD40</i>	WD40 coatomer / anthocyanin biosynthesis	(Cao et al., 2017)
Solyc09g065100.2.1	14.18672027	<i>AN1</i>	AN1-like transcription factor / anthocyanin biosynthesis	(Spelt et al., 2000)
Solyc10g084380.1.1	3.439413134	<i>PH3</i>	WRKY transcription factor / anthocyanin biosynthesis	(Verweij et al., 2016)
Solyc10g083900.2.1	3.542474125	<i>Myb27</i>	MYB transcription factor / inhibitor of anthocyanin biosynthesis	(Albert et al., 2011, 2014)
Solyc01g095640.2.1	4.928760848	<i>SITRY</i>	Trichome initiation factor ECT3 / anthocyanin biosynthesis	(Tominaga-Wada et al., 2013; Kim et al., 2017)
Solyc01g105880.4.1	3.469067651	<i>TPS4</i>	Monoterpenoid synthase / terpene biosynthesis	(Velázquez-Márquez et al., 2021)
Solyc09g092470.2.1	5.695783056	<i>TPS14</i>	Sesquiterpene synthase / terpene biosynthesis, biotic (<i>F. oxysporum</i> resistance)	(Velázquez-Márquez et al., 2021)
Solyc11g017240.2.1	3.78371117	<i>SICM2</i>	Chorismate mutase / volatile compounds biosynthesis, abiotic (drought stress)	(Tzin et al., 2015; Filiz et al., 2019)

Solyc08g008630.3.1	-3.340796451	<i>Dwarf27</i>	Beta-carotene isomerase D27 / stringolactone and beta-carotene biosynthesis, biotic (psyllid resistance)	(Harrison et al., 2021)
Solyc04g050930.3.1	-2.072953851	<i>VDE</i>	Violaxanthin de-epoxidase VDE / carotenoid biosynthesis, biotic (<i>P. syringae</i> infection), abiotic (anoxia)	(Yang et al., 2015b)
Solyc05g010180.3.1	-2.221215961	<i>CRTISO</i>	Carotenoid isomerase / carotenoid biosynthesis	(Isaacson et al., 2002)
Solyc12g006140.2.1	4.364619823	-	chlorophyll a/b-binding protein / fruits ripening	(Zouari et al., 2014)
Solyc02g065220.3.1	-2.141082227	-	Cytochrome P450 / fruits ripening (extended shelf-life)	(Gao et al., 2018)
Solyc09g066150.1.1	-4.574546484	-	Cytochrome P450 / fruits ripening (extended shelf-life)	(Gao et al., 2018)
Solyc07g006570.3.1	4.221848981	-	Ribonuclease 3-like / RNA biogenesis	(Consortium et al., 2012)
Solyc02g065230.3.1	3.170387722	<i>IMT7</i>	Cytochrome P450	-
Solyc02g091440.2.1	2.129583909	<i>bHLH83-like</i>	bHLH / enhance root hair initiation, promote flowering in short day and maintain the iron balance	(Qian et al., 2021)
Solyc12g088130.2.1	4.327213562	<i>bHLH93-like</i>	bHLH / enhance root hair initiation, promote flowering in short day	-
Solyc04g077780.3.1	2.019567752	-	LIM transcription factor / cytoskeleton organization	-
Solyc12g013850.2.1	-2.036803744	-	Glycosyltransferase / regulates anther development and male-sterility	(Omidvar et al., 2015)
Solyc02g081340.3.1	10.53476424	<i>GST</i>	Glutathione-S-transferase / anthocyanin biosynthesis	(Alfenito et al., 1998)
Solyc01g058030.2.1	8.08401203	-	Gibberellin2-beta-dioxygenase / determines dwarf phenotype with shorter internodes	(Sun et al., 2019)
Solyc05g054360.3.1	6.837513657	-	Pectin methylesterase / flower initiation	(Wen et al., 2020)
Solyc12g010500.2.1	5.358159809	-	U-box protein / biotic (resistance to <i>X. perforans</i>)	(Shi and Panthee, 2020)

4.1.1.1 GOE analysis

MF resulted the most abundantly represented GO category, with 9 GO terms and an intersection size of 15 genes over a query size of 165 genes. The most enriched terms were related to ‘enzyme inhibitory activity’, with 9 up-regulated DEGs, 7 of which are classified as serine-type endopeptidase inhibitors in tomato. Among them, Solyc03g020080.3.1, Solyc03g020030.3.1, Solyc11g020960.2.1, Solyc08g080630.3.1, Solyc10g086090.2.1, Solyc10g086100.2.1 encode specific Pin-II type proteinase inhibitors or protease inhibitors that have been already demonstrated to be players of the defence against wounding, pathogens and pests and of response to abiotic stress, especially UV, drought and heat stress in tomato (Bergey et al., 1996; Conconi et al., 1996; Fan et al., 2020). Within MF also ‘beta-glucosidase’ and ‘glucosidase activity’ terms were represented with 4 up-regulated DEGs. Among them, Solyc01g059965.1.1 has been demonstrated to be implicated in the defence of tomato plants against pathogens (Murata et al., 2019) and its expression has been found to be modulated in tomato upon infection with *Cladosporium fulvum* and *Phytophthora infestans* (van Kan et al., 1992; Fan et al., 2021). Up-regulation of Solyc06g073760.3.1 has been correlated to increased softening of transgenic tomato overexpressing a MADS-box transcription factor, affecting fruit development and ripening (Huang et al., 2017).

Within the BP category, coherently with secondary metabolites analysis, the ‘flavonoid biosynthetic process’ term was found. In particular, GOE analysis highlighted the up-regulation of 4 genes: Solyc05g053550.3.1, Solyc09g091510.3.1, Solyc02g083860.3.1, Solyc02g085020.3.1. Solyc09g091510.3.1 and Solyc05g053550.3.1 are tomato *chalcone synthase* (*CHS*) I and II, respectively, that are early pathway genes that determine the accumulation of the naringenin chalcone precursor of anthocyanins and of other flavonoids (Zhang et al., 2019). Solyc02g083860.3.1 is tomato *Flavonoid-3-hydroxylase* (*F3H*) that leads to the accumulation of dihydromyricetin, dihydrokaempferol and dihydroquercetin precursors of anthocyanins and of other flavonoids in tomato (Aoki et al., 2010; Zhang et al., 2019). Solyc02g085020.3.1 is tomato *dihydroflavonol reductase* (*DFR*) that leads to the accumulation of leucoanthocyanidins in tomato (Bongue-Bartelsman et al., 1994).

Within the CC GO category, ‘Extracellular Region’ and ‘Casparian Strip’ terms were categorized. Seventeen genes over a query size of 115, were found to be up-regulated within these terms. These genes, in many cases, are specifically correlated to lignin biosynthesis, cell wall organization and resistance to abiotic and biotic stress functions in tomato. In particular, Solyc05g052245.1.1 and Solyc09g010860.3.1 are the tomato expansins A8-like and EXPA4, involved in cell wall organization (Sato et al., 2012). Solyc02g062510.3.1, that shares 86%

homology with the *S. tuberosum* Peroxidase 72-like gene, and Solyc02g077300.2.1 (tomato Peroxidase 19) are involved in the phenylpropanoids biosynthesis (Mei et al., 2009). In addition, also Solyc04g080760.3.1 (tomato Peroxidase 9) plays a role in hypoxia tolerance and maintain the iron balance in tomato (Safavi-Rizi et al., 2020). Peroxidase 9 was also demonstrated to be a pathogenesis-related protein up-regulated upon *Tomato Mosaic Virus* infection (Andolfo et al., 2014). Solyc06g054320.1.1, Solyc10g055190.1.1, Solyc10g055200.1.1 and Solyc04g010270.1.1 represent tomato dirigent proteins that contribute to the dimerization of conyferil alcohol, a crucial step toward lignin biosynthesis, modulating cell wall metabolism during abiotic and biotic stress exposure in tomato (Paniagua et al., 2017). Solyc01g110110.3.1 and Solyc02g076710.3.1 are tomato cysteine proteinases. In particular, Solyc02g076710.3.1 shares 90% homology with *N. benthamiana* cathepsin B-like cystein proteases that are involved in the hypersensitive response (McLellan et al., 2009). Solyc08g080630.3.1, already highlighted as an up-regulated DEG within the MF category GOE, is the tomato *SIP131* protease inhibitor. This gene has been demonstrated to be up-regulated under *Tomato Spotted Wilt Virus* infection in tomato roots and leaves and has been shown to be up-regulated in drought-tolerant tomato lines and drought-sensitive varieties under drought conditions (Fan et al., 2020). GOE analysis revealed only 1 down-regulated DEG belonging to the Molecular Function term ‘glycosyl transferase’. Solyc05g053890.2.1 represents the complete sequence of the tomato UDP-GT-like which is probably related to glycosylation of flavonoids prior to their transport to the vacuole (Solyc04g016200.1.1, Solyc04g016210.3.1, Solyc05g053890.2.1, Solyc01g095760.3.1 are incomplete sequences of tomato UDP-GT-like) (Fernandez-Moreno et al., 2016).

4.1.1.2 DEGs analysis

Despite GOE analysis did not give rise to the Response to stimulus category, a relatively high number of DEGs was found that could be associated with response to abiotic and biotic stress response. 15 up-regulated and 7 down-regulated DEGs were found to be correlated with such a response. Among up-regulated DEGs involved in early signals of defence responses against environmental cues, Solyc01g096560.2.1 ($\text{Log}_2\text{Fc} = 4,10$; *TomLOXD*), was retrieved. *TomLOXD* encodes a lipoxygenase that has been demonstrated to elevate wound-induced jasmonate response, up-regulation of wound-induced genes and enhanced resistance to insects and necrotrophic pathogens in tomato (Yan et al., 2013). Solyc07g054840.3.1 ($\text{Log}_2\text{Fc} = 3.34$; tomato transcription factor 41) shares best homology with Myb41 of *A. thaliana* where it functions as a Map-kinase involved in a number of signaling pathways that control plant

development and salt stress tolerance (Hoang et al., 2012). Solyc03g095810.3.1 (Log₂Fc = 6.92; tomato Trichome birefringence-like protein) up-regulation mediates xylan acetylation and has been demonstrated essential in tomato for invading microorganism resistance and against environmental stress like cold and drought (Zhang et al., 2020). Solyc10g007970.2.1 (Log₂Fc = 6.96; tomato WRKY transcription factor 77) has been demonstrated to be involved in the signalling to water deficit in tomato (Asins et al., 2021). Solyc07g043690.2.1 (Log₂Fc = 2.80; tomato *SINPRI*) has been suggested to regulate tomato plant drought response (Li et al., 2019). Solyc03g096460.3.1 (Log₂Fc = 2.30) is a known modulator of wound signalling in tomato (Scranton et al., 2013). Solyc02g080790.3.1 (Log₂Fc = 3.31; tomato deoxypusine synthase *SIDHS*) has been shown to be up-regulated during osmotic stress and chilling injury (Wang et al., 2001; Gupta et al., 2013). Solyc10g081300.1.1 (Log₂Fc = 2.56; *SIMC8*), encodes a metacaspase that is up-regulated during apoptosis induction by pests and regulated by drought, cold and salt in tomato (Liu et al., 2016). Solyc10g080690.2.1 (Log₂Fc = 2.30; tomato patatin defence protein) has been demonstrated to be up-regulated upon soil flooding in tomato (De Ollas et al., 2021). Solyc06g073760.3.1 (Log₂Fc = 4.07; tomato β 1,3- glucanase 2 *BGL2*) has been found to be negatively correlated with the sugar/organic acid ratio of tomato fruits (Li et al., 2021). The simultaneous up-regulation of *BGL2* and *PRI*, the marker genes of Salicylic Acid (SA) pathway, is a hallmark of systemic resistance induced in tomato plants against different pathogens and can be followed by accumulation of SA at high levels (Peng et al., 2004; Fahim et al., 2016; Hanan et al., 2020). Solyc12g035225.1.1 (Log₂Fc = 9.26; tomato putative zinc-finger domain-containing protein) shares 75% homology with rice *RICESLEEPER1* that was found to be up-regulated in *S. chilense* in relation with transcription factors for salt tolerance (Kashyap et al., 2020). Solyc12g010500.2.1 and Solyc12g010670.1.1 (Log₂Fc = 5.35 and 5.36, respectively) are categorized as E3 ubiquitin proteins that have been found to be up-regulated in tomato genotypes resistant to *Xanthomonas perforans* (Shi and Panthee, 2020). Solyc12g009630.2.1 (Log₂Fc = 5.18; tomato calmodulin *SlCaM3*), encodes an important calcium-binding protein which has been found to be up-regulated in tomato stem and roots upon *Botrytis cinerea* infection and mechanical wounding (Peng et al., 2014). In addition, it has been reported that *SlCaM3* is strongly expressed under salt and cold stress in tomato (Shi and Du, 2020). Solyc02g067750.3.1 (Log₂Fc = 6.91) shares 80% identity with *N. benthamiana* *CA1*. During drought stress CA1 proteins gradually diminish within chloroplast and are accumulated in the cytosol, suggesting that they could be translocated from chloroplasts to the cytosol and act as a signal messenger from the chloroplast in tomato (Li et al., 2020a). Importantly, in view of the possible improvement of resistance to abiotic stresses relevant to space, Solyc08g075705.1.1

(Log₂Fc = 4.25; tomato plastidial thioredoxin Y2, *pTRXy2*) was found to be up-regulated. PTRX y2 together with pTRX y1 and x-type TRXs, is mostly involved in ROS detoxification and take part to the complex redox signaling network regulating tomato plant development (Serrato et al., 2013).

Among down-regulated DEGs correlated with resistance functions, Solyc09g007190.3.1 (Log₂Fc = -2.21; tomato peroxyredoxin-like 2A PRXL2A) redox regulatory protein was found. Solyc03g098760.2.1 (Log₂Fc = -5.10; tomato proteinase inhibitor I3 Kunitz-type trypsin inhibitor) has been demonstrated to be up-regulated upon the cell-content feeding mite *Tetranychus* species infection (Schimmel et al., 2018) and it is told to protect seeds from predators (Islam et al., 2015). Solyc09g089490.3.1 (Log₂Fc = -5.21; tomato SIPI40 protease inhibitor) can be induced by abiotic (drought and salt) and biotic (*Botrytis cinerea* and *Tomato Spotted Wilt Virus*) stress (Fan et al., 2020). Solyc06g008760.1.1 (Log₂Fc = -3.05; tomato *Glutharedoxin-C13-like*) has been demonstrated to be upregulated in ripening inhibitor (*rin*) mutants of *S. lycopersicum* (Kumar et al., 2016). Solyc07g055610.2.1 (Log₂Fc = -2.05); tomato Resistance protein R1) has been related to resistance to late blight (Bournival et al., 1989) and it has been shown to be linked to proteinase inhibitor I3 upon *Fusarium oxysporum* infection (Bournival et al., 1989; Pan et al., 2000). Solyc01g010480 (Log₂Fc = -2.2; tomato K⁺ channel KAT1) was demonstrated to be highly up-regulated in low K tolerant tomato genotypes upon potassium deficiency (Zhao et al., 2018). Solyc01g102610.3.1 (Log₂Fc = -3.60; tomato FRO6) is involved in nutrient transport in phloem and was observed to be down-regulated under *Phytoplasma solanii* infection of tomato (Buoso et al., 2019).

Despite GOE retrieved only 4 genes among ‘flavonoid biosynthetic process’ term, many other up-regulated DEGs resulted in the analysis that are associated with biosynthesis of anthocyanins and of other specialized metabolites (Figure 4.3). Twelve additional structural genes and 6 transcription factors were identified as up-regulated DEGs. In addition to *CHS*, *F3H* and *DFR*, DEGs analysis revealed also tomato naringenin-chalcone isomerase (*CHI*) (Solyc05g010320.3.1; Log₂Fc = 4.30 and Solyc05g052240.3.1; Log₂Fc = 8.45), tomato flavonoid-3',5'-hydroxylase *F3'5'H* (Solyc11g066580.2.1; Log₂Fc = 16.35), tomato anthocyanin synthase *ANS* (Solyc08g080040.3.1, Log₂Fc = 8.86) were found. Interestingly, also Solyc04g078140.3.1, which shares best homology with *Cytochrome b5* of *P. hybrida*, where it is essential for full activity of *F3'5'H*, was up-regulated (Log₂Fc = 8.35) (De Vetten et al., 1999). In addition, tomato *caffeoyl-CoA-O-methyltransferase* (Solyc09g082660.3.1; Log₂Fc = 15.0503), already identified as the prime candidate gene responsible for anthocyanin methylation in tomato due to significant correlation of expression with *ANS*, *DFR* and *F3'5'H* in *Roseal* and

Delila fruits (Roldan et al., 2014), resulted up-regulated. It shares best homology with *P. hybrida* O-methyltransferase (*OMT*). Interestingly, it was found to have significant correlation with abiotic stress in tomato (Shi and Panthee, 2020).

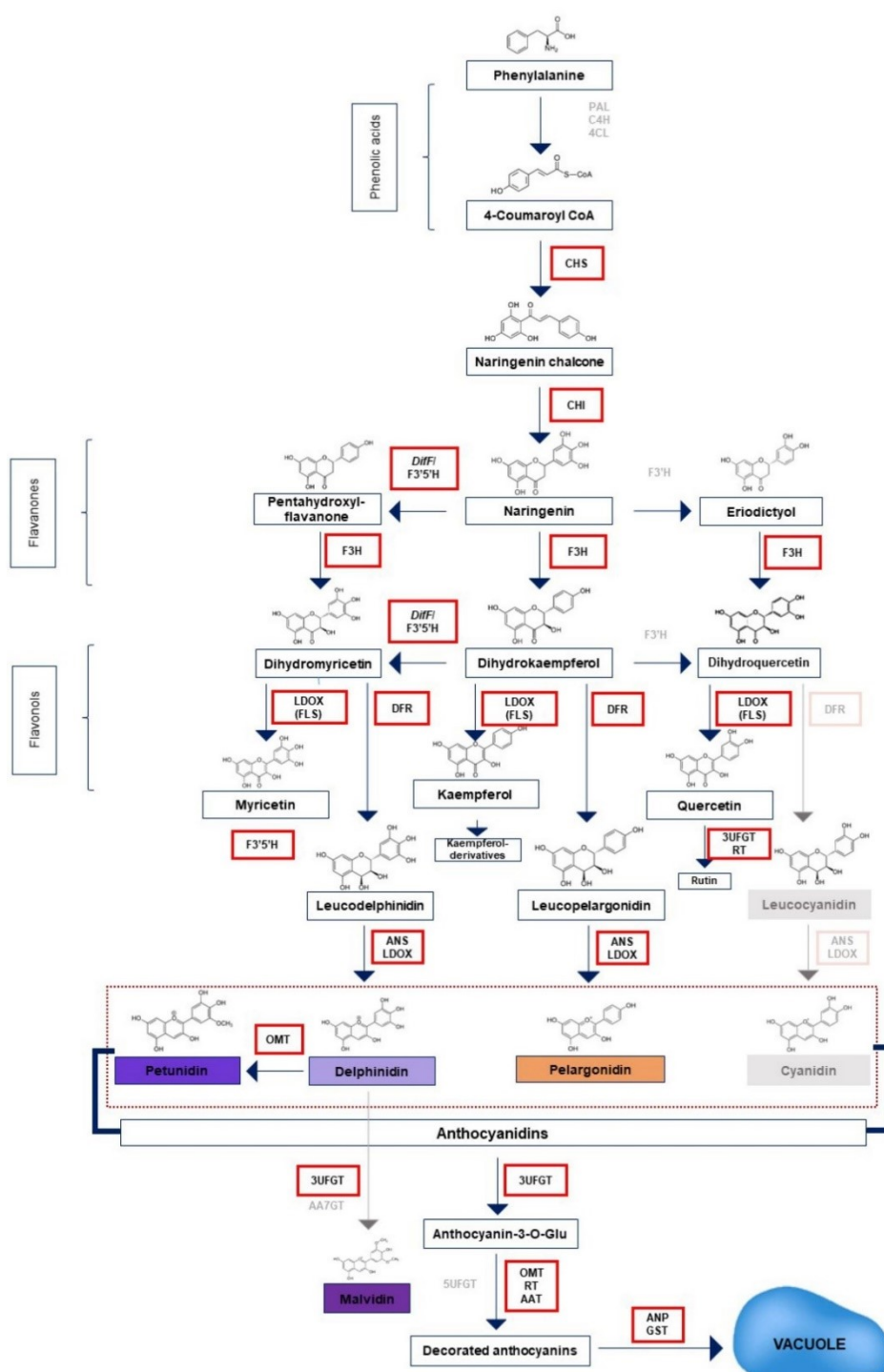


Figure 4. 3 Schematic diagram of the anthocyanin biosynthetic pathway. The structural genes up-regulated by ectopic expression of *PhAN4* in MicroTom HRCs and leading to accumulation of compounds are in black and marked by red boxes. PAL, phenylalanine amoni-aliase; C4H, cinnamate-4-hydroxylase; 4CL, 4-coumarate CoA ligase; CHS, chalcone synthase; CHI, chalcone isomerase; F3'H, flavonoid-3'-hydroxylase; F3'5'H, flavonoid-3',5'-hydroxylase; DFR, dihydroflavonol reductase; ANS, anthocyanin synthase; OMT, O-methyltransferase; AAT, anthocyanin acyltransferase; RT, rhamnosyltransferase; UFGT, UDP-glucose flavonoid 3-O-glucosyl transferase; AA7GT, cyanidin-3-O-glucoside-7-O-glucosyltransferase; ANP, anthocyanin permease; GST, glutathione-S-transferase.

Three tomato UDP-glucose flavonoid 3-O-glucosyl transferases (*3UGT*) (Solyc02g062975.1.1, $\text{Log}_2\text{Fc} = 7.08$; Solyc10g083440.1.1, $\text{Log}_2\text{Fc} = 9.97$; Solyc12g098590.2.1, $\text{Log}_2\text{Fc} = 16.55$) catalyzing the transfer of the glucosyl moiety from UDP-glucose to the 3-hydroxyl group of anthocyanidins in tomato were found among up-regulated DEGs, as well (Hu et al., 2011; Tohge et al., 2020). Furthermore, anthocyanidin-3-O-glucoside rhamnosyltransferase (*3RT*) (Solyc09g059170.2.1, $\text{Log}_2\text{Fc} = 8.3262$), that in tomato controls the conversion of anthocyanidin-3-glucosides to anthocyanidin-3-rutinosides by the UDP rhamnose, was found among up-regulated DEGs (Tohge et al., 2020). Tomato anthocyanin acyltransferase (*AAT*, or Flavonoid-3-O-rutinoside-4'''-O-phenylacyltransferase; Solyc12g088170.2.1, $\text{Log}_2\text{Fc} = 13.21$) resulted up-regulated, as well. Solyc02g081340.3.1 ($\text{Log}_2\text{Fc} = 10.53$) resulted among up-regulated DEGs. In tomato it encodes a putative Glutathione S-Transferase that shares 84% homology with *P. hybrida* *GST* that is responsible of anthocyanin sequestration in the vacuole (Alfenito et al., 1998). Solyc03g025190.4.1 ($\text{Log}_2\text{Fc} = 16.80$; tomato *MTP77*) resulted highly up-regulated among DEGs. This gene belongs to clade 1 of the multidrug and toxic compound extrusion (MATE) family member (*Transparent testa 2-like*), that in tomato has been associated with vacuolar chloride channels related to regulation of cell turgescence. In MicroTom, many clade 1 MATE have been functionally related to the transport of secondary metabolites (dos Santos et al., 2017). Solyc10g006120.2.1 ($\text{Log}_2\text{Fc} = 7$) was found to be up-regulated, as well. This gene shares 59% sequence identity with leucoanthocyanidin dioxygenase (*LDOX*) from *P. hybrida* and 61% with *S. tuberosum* *FLS* (Pelletier et al., 1997). *LDOX* has been demonstrated to be a bi-functional enzyme being able both to convert leucoanthocyanidins into anthocyanidins and to catalyze the in planta formation of flavonols in *fls1-2* mutants of *A. thaliana* (Preuß et al., 2009).

Together with regulation of structural genes, *PhAN4* positively modulated also transcription factors involved in anthocyanin biosynthesis. Solyc07g052490.3.1 ($\text{Log}_2\text{Fc} = 7.17$, tomato Myb-like transcription factor *Atv*) is involved in anthocyanin biosynthesis (Cao et al., 2017). Also, Solyc10g086290.2.1 ($\text{Log}_2\text{Fc} = 8.65$), that shares 83% homology with *Solanum tuberosum* *AN2-like*, that is a R2R3Myb involved in anthocyanin biosynthesis, was found (Colanero et al., 2020; Sun et al., 2020; Yan et al., 2020). Solyc09g065100.2.1 ($\text{Log}_2\text{Fc} = 14.18$) maps the Hoffman's Anthocyaninless (AH) locus and encodes a bHLH factor (SIAN1) that positively regulates anthocyanin biosynthesis in tomato (Qiu et al., 2016). No effects of *PhAN4* expression were found on another known regulator of anthocyanins biosynthesis, *AN11*. Also Solyc10g084380.1.1 was up-regulated ($\text{Log}_2\text{Fc} = 3.43$). In *Solanum lycopersicum* this gene

encodes a WRKY that shares 76% homology with *PH3* from *Petunia hybrida* where it regulates vacuolar acidification and boosts anthocyanin biosynthesis, as well (Verweij et al., 2016).

Among up-regulated DEGS putative repressors of anthocyanins biosynthesis were found. The up-regulated Solyc12g005800.2.1 ($\text{Log}_2\text{Fc} = 7.51$) is the tomato SIMYBATV-like (Cao et al., 2017), an R3-MYB repressor. Solyc10g083900.2.1 ($\text{Log}_2\text{Fc} = 3.54$; tomato R2R3Myb transcription factor 27) shares the best homology with *Myb27* from *P. hybrida*, where it is a repressor of the synthesis of anthocyanins (Albert et al., 2011, 2014) and Solyc01g095640.2.1 ($\text{Log}_2\text{Fc} = 4.92$; tomato *ETC3* or *SITRY*) orthologous in *A. thaliana* acts a repressor of anthocyanins accumulation, as well (Tominaga-Wada et al., 2013).

To complete the description related to the modulation of genes related to specialized metabolites, three DEGs resulted to be up-regulated and three down-regulated. Among up-regulated DEGS, Solyc01g105880.4.1 and Solyc09g092470.2.1 ($\text{Log}_2\text{Fc} = 3.46$ and 5.69 , respectively; tomato monoterpenoid synthases 2 *TPS4* and *TPS14*), are involved in the biosynthesis of monoterpenes and sesquiterpenes, respectively. *TPS4* has been found to be up-regulated in tomato cultivars resistant to *Fusarium oxysporum* (Velázquez-Márquez et al., 2021). Solyc11g017240.2.1 ($\text{Log}_2\text{Fc} = 3.78$; tomato chorismate mutase 2 *SICM2*), catalyzes the first step of the shikimate pathway from phenylalanin to the volatile compounds responsible for tomato fruit aroma and quality and defense from biotic and abiotic stress response (Tzin et al., 2015). In tomato, *SICM2* has been showed also to be up-regulated under drought stress (Filiz et al., 2019). Among down-regulated genes, Solyc08g008630.3.1 ($\text{Log}_2\text{Fc} = -3.34$; tomato chloroplastic beta-carotene isomerase D27 *Dwarf27*) is involved in stringolactone and beta-carotene biosynthesis and has been found to be down-regulated in psyllid-infested tomato plants (Harrison et al., 2021). Solyc04g050930.3.1 ($\text{Log}_2\text{Fc} = -2.07$; tomato violaxanthin de-epoxidase *VDE*) is involved in the carotenoid biosynthesis. Suppression of *VDE* can induce the photo-inhibition of the PSII and, at the same time, it results in an accumulation of fucoxanthin that functions as an efficient anti-oxidant in anoxia conditions (Tohge et al., 2020). Moreover, it has been shown that *VDE* is down-regulated in tomato plants upon *Pseudomonas syringae* pv. tomato DC3000 infection (Yang et al., 2015b). Solyc05g010180.3.1 ($\text{Log}_2\text{Fc} = -2.22$; tomato carotenoid isomerase *CRTISO*) is involved in carotenoids biosynthesis. In MicroTom fruits, the down-regulation of carotenoid isomerase has been demonstrated to induce an accumulation of zeta-carotene and cis-prolycopene (dos Santos et al., 2017; Florio et al., 2021), both elevating and modifying carotenoid profiles towards more bioavailable forms compared to wild-type (Pelletier et al., 1997).

To complete the description related to DEGs that were modulated upon expression of *PhAN4* and that were related to plant physiology, in addition to Solyc06g073760.3.1 (already described in GOE analysis in the MF category), also Solyc12g006140.2.1 (Log₂Fc = 4.36; tomato chloroplastic light-harvesting chlorophyll a/b-binding protein 37) was found to be up-regulated. It is involved in tomato plants photosynthesis and in ripening fruit metabolism (Zouari et al., 2014). Among down-regulated DEGs, Solyc02g065220.3.1 (Log₂Fc = -2.14; tomato cytochrome P450) in VIGs *Del/Ros* tomatoes was showed to have significant difference in transcript levels between purple and red sectors during late ripening and its down-regulation has been associated to extended shelf life (Gao et al., 2018). Solyc09g066150.1.1 (Log₂Fc = -4.57; putative tomato cytochrome P450) suppression has been found in transgenic tomato plants unable to perform DNA methylation and its up-regulation has been associated with fruit ripening (Zhao et al., 2018).

4.1.2 Analysis of AN4 HRCs phenylpropanoids

Total anthocyanins content was measured in AN4 HRCs showing concentrations equal to $37 \pm 1.85 \mu\text{g/g}$ dried weight in the best clone (AN4-1; Figure 4.4, left).

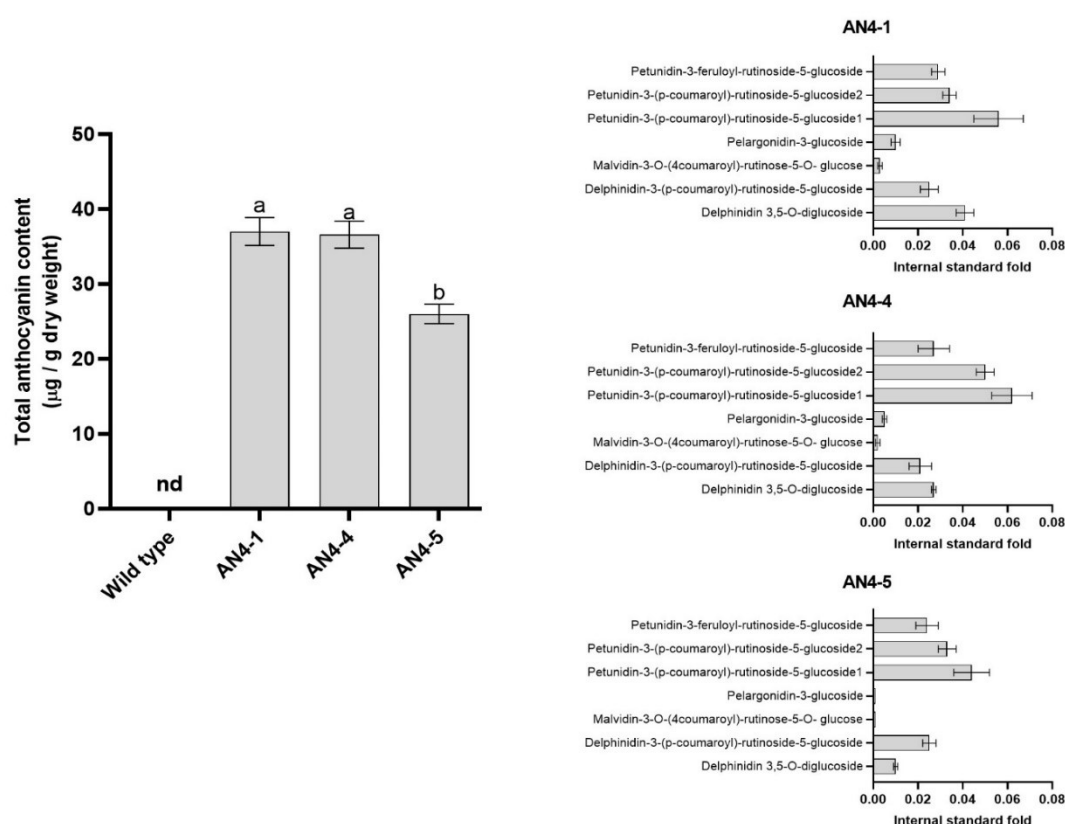


Figure 4. 4 Anthocyanins in AN4 HRCs clones. Total anthocyanins content (left) and amount of specific anthocyanins (right) of three representative *PhAN4*-expressing hairy root clones. Columns report average values $\pm$ s.d. (n=3).

The anthocyanin profile of AN4 HRCs clones was determined by LC-HRMS and compared to control. Identification was achieved by *m/z* ion reconstruction starting with the aglycon (delphinidin, petunidin, pelargonidin, malvidin), followed by the recognition of all the conjugated sugar and phenolic moieties. Subsequently, absolute quantification was performed as previously described (Carmona et al., 2021) and by interpolating anthocyanin signal intensities in the roots compared to the ones of external calibration curves of the Pelargonidin-3-glucoside and Delphinidin 3,5-O-diglucoside standards.

In our experimental conditions, anthocyanins were virtually undetectable in control HRCs. Petunidin-3-(*p*-coumaroyl)-rutinoside-5-glucoside¹ and Delphinidin 3,5-O-diglucoside were the most abundant anthocyanins in the AN4 roots, followed by a second group including Petunidin-3-(*p*-coumaroyl)-rutinoside-5-glucoside², Petunidin-3-feruloyl-rutinoside-5-glucoside and Delphinidin-3-(*p*-coumaroyl)-rutinoside-5-glucoside. Two additional anthocyanins, Pelargonidin-3-glucoside and Malvidin-3-O-(4'''coumaroyl)-rutinose-5-O- glucose, were detected, although at low levels (Figure 4.4, right) (Su et al., 2016). All the clonal lines showed about the same relative amount of the different anthocyanin species.

To evaluate the effect of the accumulation of anthocyanins on their precursors and on the final balance of flavonoids accumulation, a detailed analysis on phenylpropanoids was carried out by LC-HRMS on the engineered HRCs. A graphical representation of the accumulation levels of anthocyanins precursors is shown for the best anthocyanin-accumulating AN4-1 HRC (Figure 4.5 and Figure 4.6). A series of phenolic acids and their derivatives (e.g., dicaffeoylquinic, 5-caffeoyl-quinic and 4-caffeoyl-quinic acids) were accumulated at a significantly lower level compared to control. This finding might be ascribed to the role of these compounds as flavanones and flavonols precursors of their sugar-decorated derivatives and of anthocyanins that, in turn, resulted to be enhanced in accumulation in engineered HRCs. Hydroxycinnamic acid as well accumulated at reduced levels in engineered HRCs, with respect to control. Interestingly, hydroxybenzoic acid level was not significantly different between engineered and control HRCs, due to contribution to different pathways than anthocyanins. Significantly higher levels of other valuable phenolics such as coumaric acid and caffeic acid derivatives were found in AN4-1 HRC compared to control (Figure 4.5). Accordingly, among flavonoids, both flavanones (i.e., naringenin chalcone and its sugar-decorated derivatives, naringenin, eriodictyol) and flavonols (i.e., dihydrokaempferol, sugar-decorated kaempferol, dihydroquercetin, quercetin derivatives, rutin and myricetin) resulted in an overall significantly higher accumulation in engineered HRCs compared to control (Figure 4.6). Coherently with phenolic acids precursor function, the observed fold change levels on most flavonoid group (from naringenin and kaempferol

derivatives to quercetin derivatives and rutin) displayed a much larger extent (192.82 ± 39.1 for naringenin dihexose I) compared to the phenolic acid precursor (0.623 ± 0.069 for 4-caffeoyl-quinic acids).

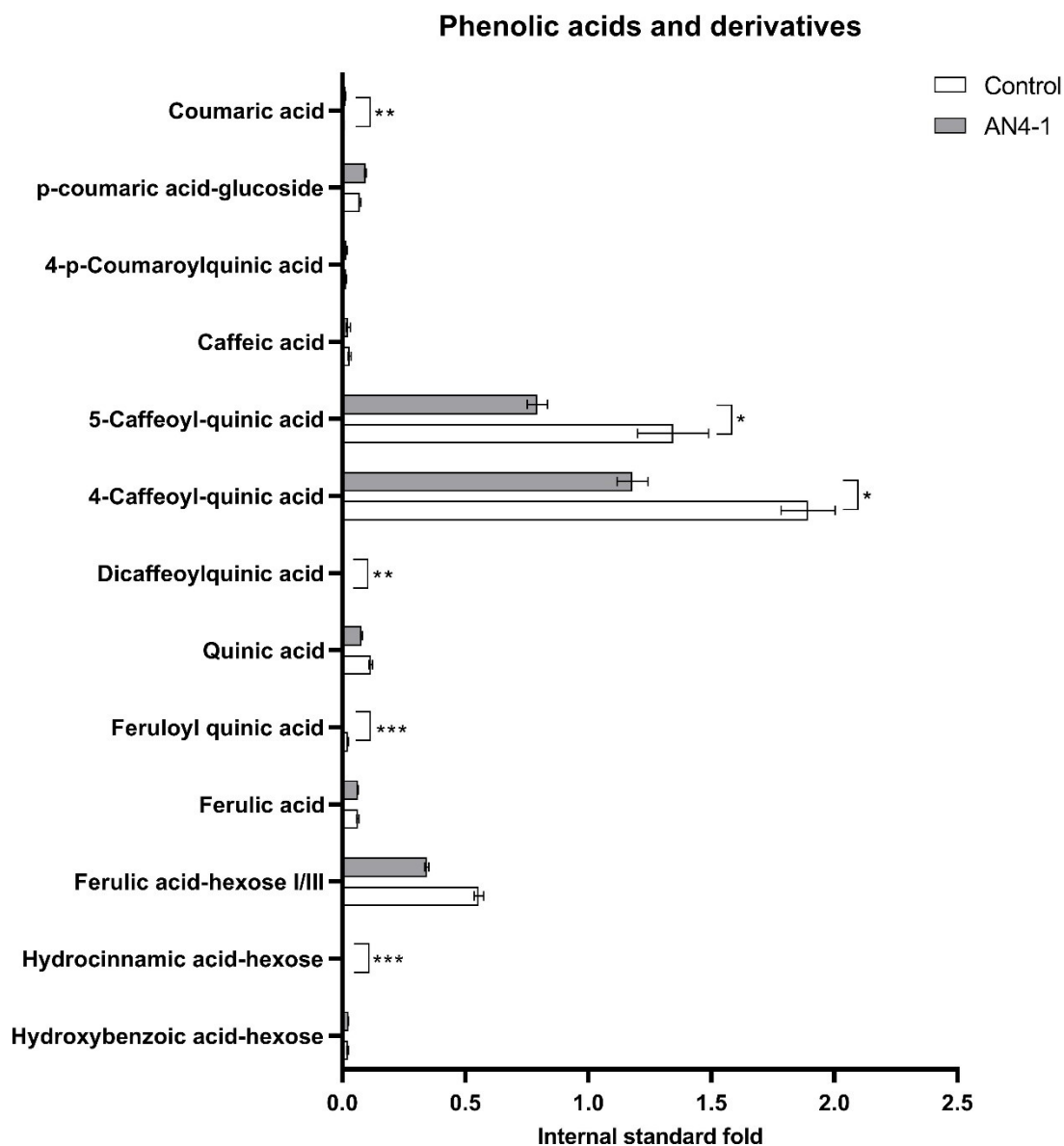


Figure 4. 5 Graph representing the level of phenolic compounds and their derivatives expressed as internal standard (formononetin) fold in control and AN4-1 HRCs. Columns report average values $\pm$ s.d. (n=3).

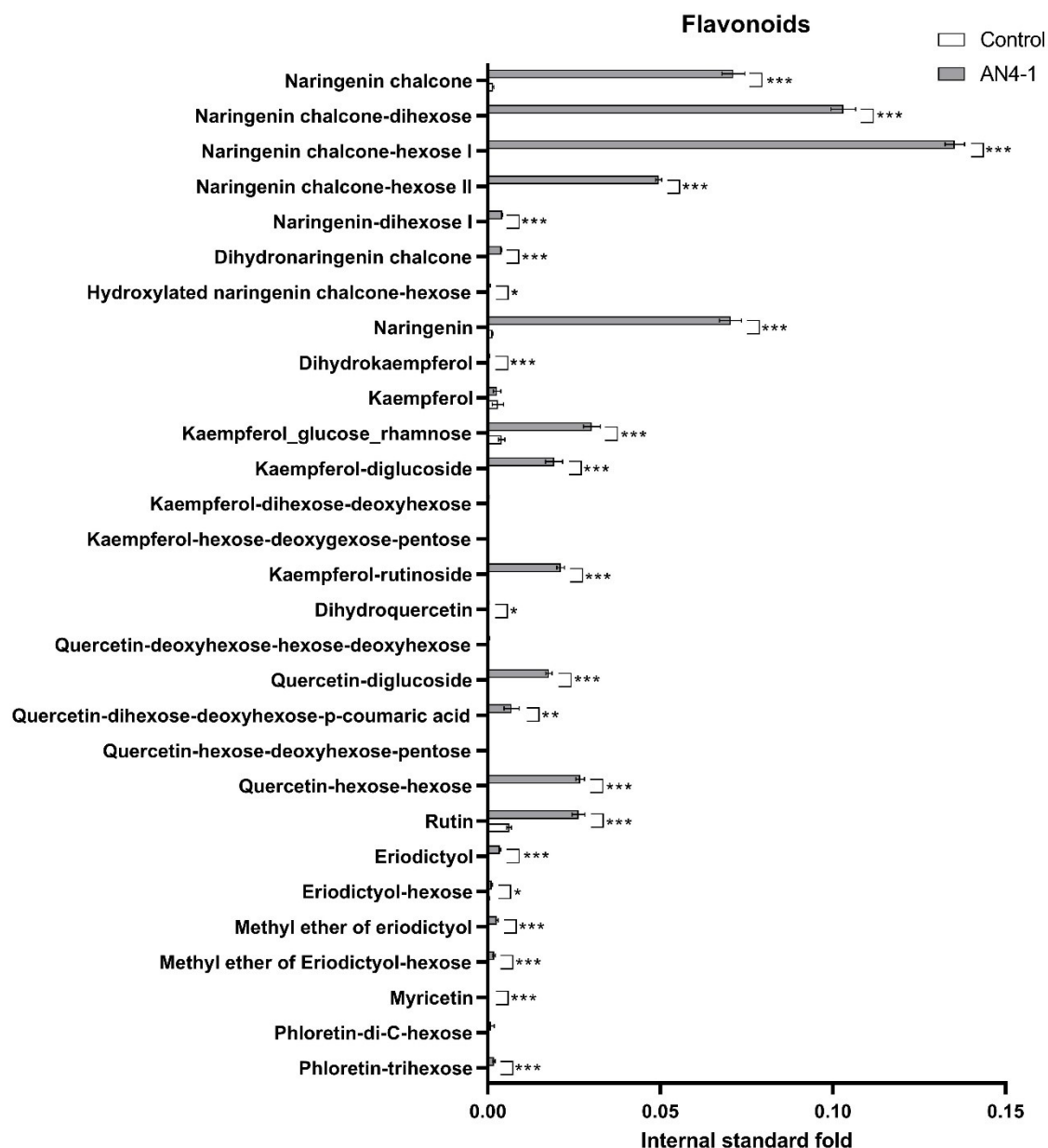


Figure 4. 6 Level of flavonoids expressed as internal standard (formononetin) fold of control and AN4-1 HRCs. Columns report average values $\pm$ s.d. (n=3).

4.1.3 Phenolic content and antioxidant (DPPH) activity

To verify whether *PhAN4* expression was associated to increased antioxidant activity, *in vitro* scavenging activity of the hydroalcoholic soluble fraction of HRCs was assessed by non-enzymatic 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay (de Torre et al., 2019).

The total phenolic content expressed as μg of gallic acid equivalents (GAE) / g of dry weight (DW) was found to be nine--fold compared to control ($18.2 \times 10^3 \mu\text{g}$ GAE / g DW in AN4-1; Figure 4.7 A).

The trolox equivalent antioxidant capacity of the hydroalcoholic soluble fraction (containing anthocyanins) in AN4 HRCs appears to be thirty times higher in the AN4-1 clone than in control (Figure 4.7 B).

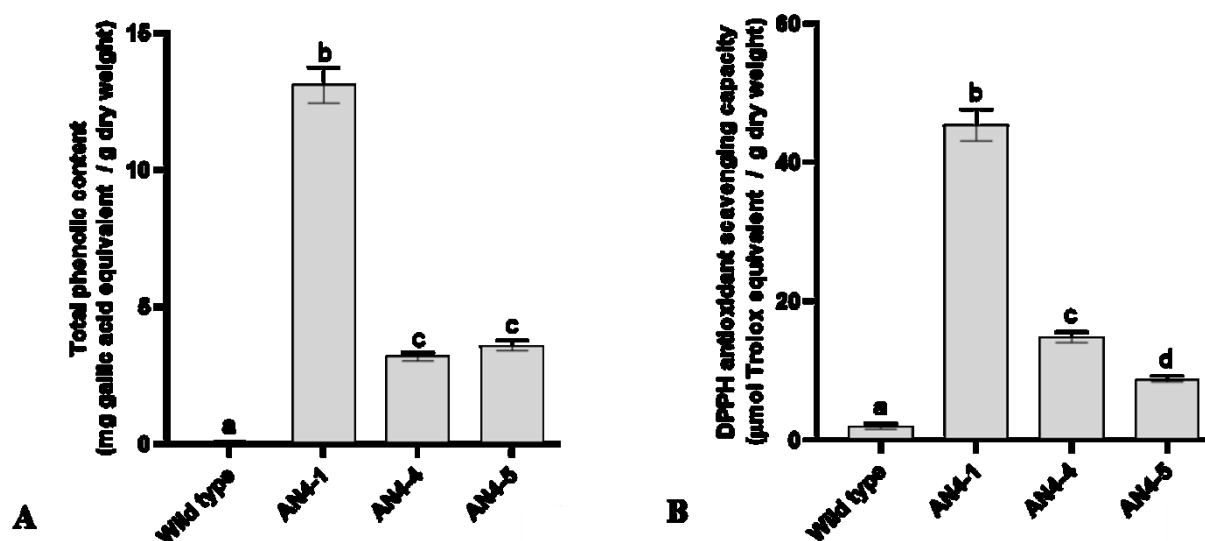


Figure 4. 7 Total phenolic content in MicroTom transgenic HRCs reported in mg of gallic acid equivalents / g of dry weight (A). Analysis of antioxidant activity (DPPH) in MicroTom transgenic HRCs reported as μg Trolox equivalents / g dry weight (DW) (B). Each analysis consisted of triplicate measurements of each sample and data were averaged over the three measurements. A univariate statistical analysis based on t-Student test and one way-ANOVA ($P \leq 0.05$) was carried out.

4.1.4 ESR analysis

To further characterize the effect of the ectopic expression of *PhAN4*, MicroTom HRC lyophilized material was investigated for its ability to counteract the generation of reactive oxygen species (ROS) and for the maintenance of this feature after high dose gamma radiation. The AN4-1 HRC was chosen as the best candidate to evaluate resistance to radiation by ESR. This analysis assessed the amount of peroxy radicals in lyophilized HRCs. The AN4-1 and control spectra before gamma radiation exposure did not show significant peaks. On the contrary, typical ESR spectra of irradiated HRCs showed specific signals (as shown in Figure 4.8 A after 2 kGy absorbed dose). Indeed, after gamma irradiation-related pro-oxidant stimulus, both control and AN4 MicroTom HRCs ESR signal was characterized by a singlet signal at 3454 Gauss ($g = 2.014$), corresponding to peroxy radicals induced by gamma irradiation (Andersen et al., 2000; Ichikawa et al., 2001; Nagata et al., 2003; Rossetto et al., 2007; Esatbeyoglu et al., 2014; Faure et al., 2014). Negligible satellite peaks below 3440 and above 3480 Gauss are present as well, and are related to several different contributions (such as cellulose-like molecules and

anthocyanins) (Andersen et al., 2000; Tuner and Korkmaz, 2007; Petrisor et al., 2008; Esatbeyoglu et al., 2014; Faure et al., 2014).

Focusing on peak intensity, after gamma irradiation (0.5, 1, 2 kGy absorbed doses), control showed a signal intensity significantly higher than AN4-1 (Figure 4.8 A, Figure 4.8 B). In particular, AN4-1 shows only a negligible signal increase after irradiation from the lowest to the highest absorbed dose (Figure 4.8 B). On the contrary, control HRC shows a constant and nearly linear increase of singlet intensity, already evident at low absorbed doses.

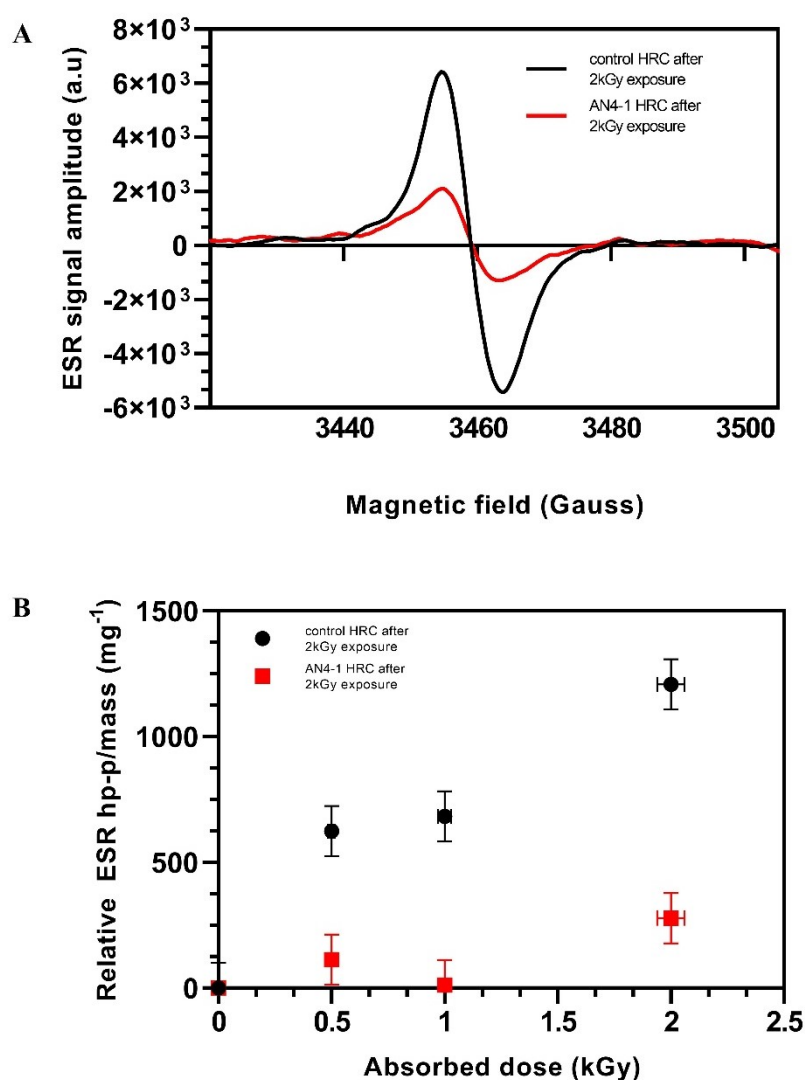
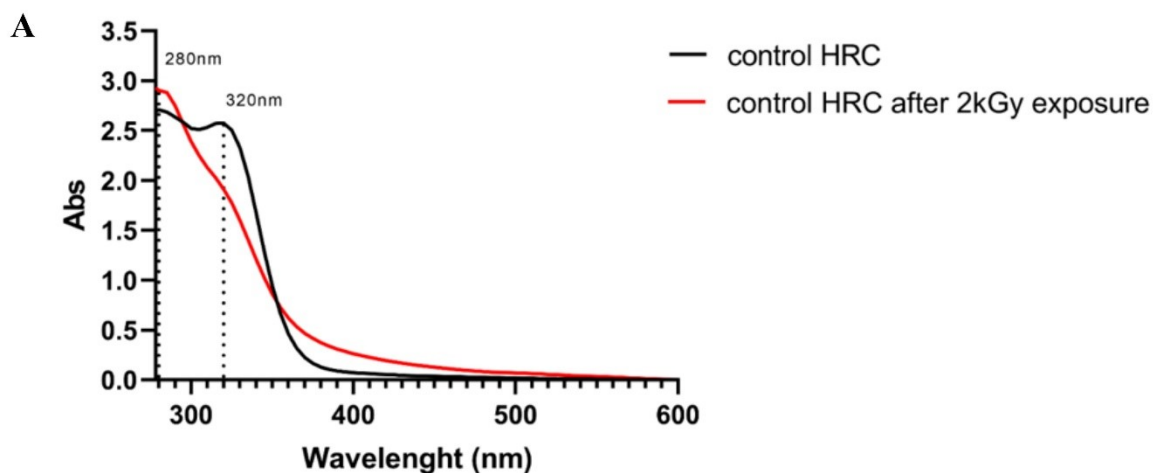


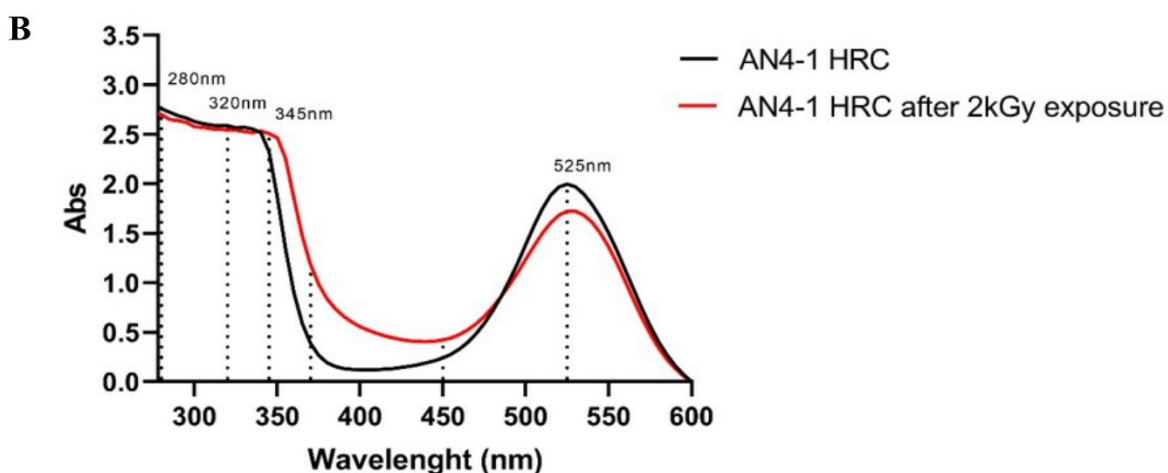
Figure 4. 8 ESR spectra of AN4-1 and control HRCs gamma irradiated at 2 kGy absorbed dose (dose rate = 1.8) (A). ESR kGy water/h signal intensity of white and purple HRCs gamma are reported as a function of the absorbed dose (up to 2 kGy absorbed dose; dose rate = 1.8 kGy water/h) (B). The intensity of each signal is expressed as the peak-to-peak height normalized for mass unit and by subtracting the intensities of the HRCs signals before irradiation.

4.1.5 UV-VIS absorbance spectra

We have used UV-VIS analysis of HRC extracts to characterize the resistance to gamma irradiation of the phenolic compounds accumulated in the AN4-1 line. Before irradiation, both control and AN4-1 acidic extracts revealed a main peak at 280 nm, followed by another peak at 315 – 320 nm (Figure 4.9).



Wavelength Bef. Irr.	Wavelength After Irr.	Abs Bef. Irr.	Abs After Irr.	Δ Abs After – Before Irr.
280 nm	290 nm	2.70	2.90	+ 0.20
315-320 nm	325 nm	2.57	2.01	- 0.56



Wavelength Bef. Irr.	Wavelength After Irr.	Abs Bef. Irr.	Abs After Irr.	Δ Abs After – Before Irr
280 nm	285 nm	2.75	2.69	- 0.06
315-320 nm	320–325 nm	2.58	2.55	- 0.03
345 nm	350 nm	2.32	2.50	+ 0.18
525 nm	525-530	1.99	1.72	- 0.27

Figure 4. 9 UV-VIS spectra of MicroTom HRCs before and after 2 kGy absorbed dose. In control, peaks at 280 nm and 320 nm indicate the presence of flavonols, hydroxycinnamic acids, tannins and flavonols (A). In addition to these, in AN4-1, the peak at 345 nm completes the flavonols group profile compared to control, while the peak at 525 nm represents anthocyanins. The curve in the region between 400 and 450 nm refers to possible glycosylation, precipitation, complexation of anthocyanins (B).

In accordance with metabolomic data, in the spectra obtained in absence of irradiation, peaks around 280 nm and 320 nm indicate the presence of flavonols, hydroxycinnamic acids, tannins and flavonols (Solis-Oviedo and De La Cruz Pech-Canul, 2019). The shoulder at 345 nm together with that at 280 nm further define of the UV-VIS spectra of the flavonols in AN4-1 (Saha et al., 2021). Anthocyanins and anthocyanins associated to phenolic acids also produce peaks around 280 nm and 320 nm, respectively (Solis-Oviedo and De La Cruz Pech-Canul, 2019), contributing to the profile of AN4-1 HRCs in Figure 4.7B. Anthocyanins result in an additional characteristic peak at 525 nm (Vivar-Quintana et al., 2002; da Silva et al., 2007; Fedenko et al., 2017) which, as expected, is observed in AN4-1 and not in control HRCs. Gamma irradiation determined a slight absorbance increase at 280 nm and a significant decrease at 320 nm in controls. On the contrary, these peaks remained unchanged in AN4-1. In AN4-1, the absorbance at 400 - 450 nm after 2kGy exposure is possibly an indication of glycosylation, precipitation, complexation with tannins of anthocyanins upon irradiation (Saha et al., 2021). Gamma irradiation determined a slight decrease of the absorbance at 525 nm in AN4-1, as confirmed by a slight discoloration of the root material after irradiation after visual inspection (data not shown).

4.1.6 Photoluminescence analysis

Photoluminescence emission spectra were analyzed to determine the resistance to misfolding and oxidation of MicroTom HRCs soluble proteins after gamma irradiation. Photoluminescence spectra of extracts containing soluble proteins from control (Figure 4.10A) and AN4-1 HRCs (Figure 4.10B) were produced. The mass-normalized emission spectra of not irradiated soluble protein samples, is similar in controls and AN4-1, and are characterized by high peaks mainly corresponding to tryptophan (370 nm) (Yang et al., 2015a; Hilaire et al., 2017) and its metabolic products such as kynurenine (470 nm) and 3-hydroxykynurenine (439 nm) (Lohmann et al., 1988; Daly et al., 2009; Gakamsky et al., 2017). In addition, a peak at 470 nm is indicative of the presence of carbamate anions, as a possible contribution by degraded amines (Pan et al., 2013). In control HRC, the 2 kGy absorbed dose caused a decrease of fluorescence emission intensity for all the three peaks. In particular, the $\Delta\%$ photoluminescence intensity after and before the

irradiation, showed that the peak at 370 nm is reduced by 67.87%, the peaks at 439 nm and 470 nm by 37.5% and 28.57%, respectively (Figure 4.10A). In AN4-1 only the 370 nm peak decreased (- 27.64%), while a slight increase (+ 18.57%) of the kynurenine peak (470 nm) was present. No significant modifications were observed at 439 nm (Figure 4.10B).

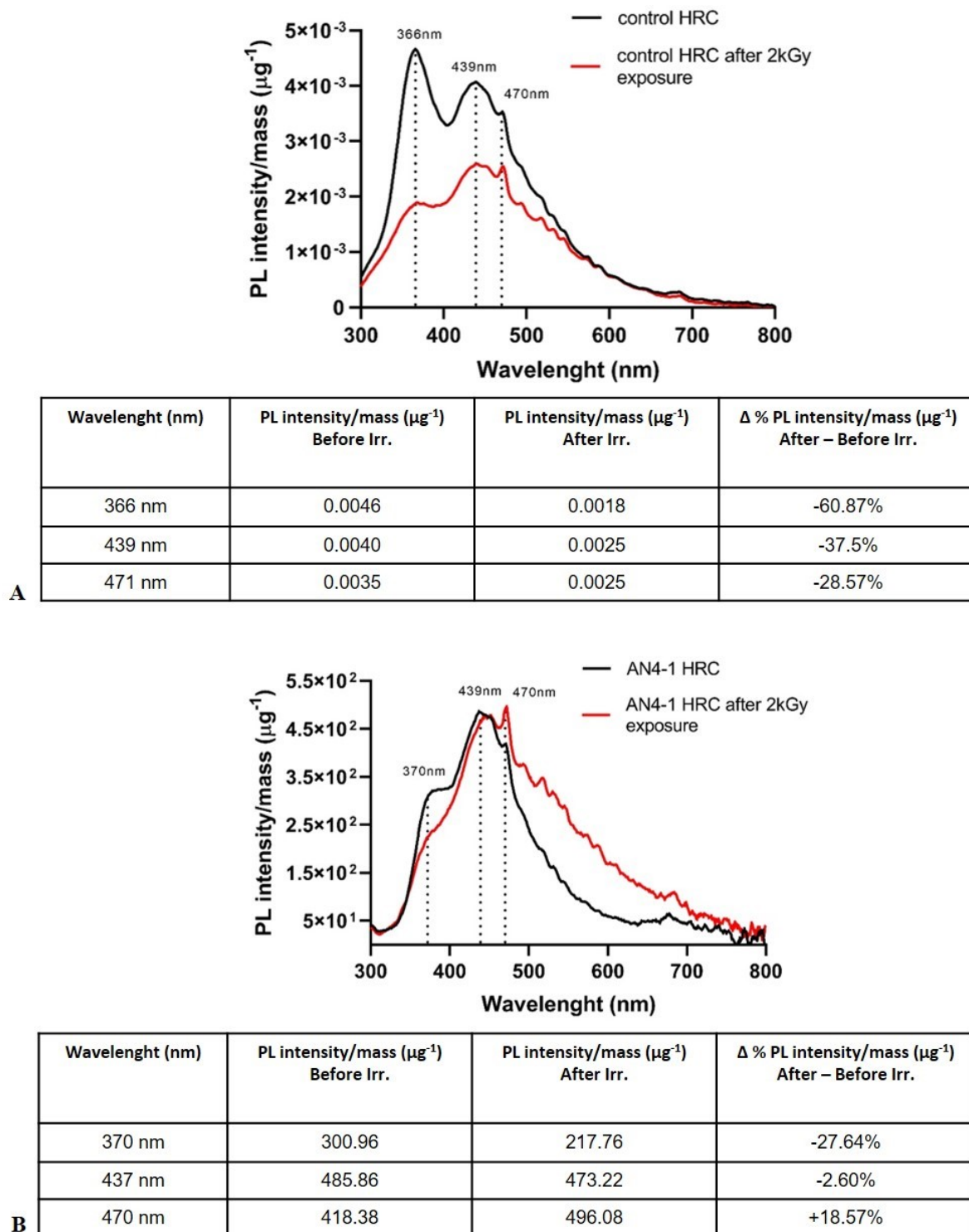


Figure 4. 10 Photoluminescence spectra of control (**A**) and AN4-1 (**B**) HRCs and related mass-normalized emission intensities (tables) before and after 2 kGy gamma irradiation. Main peaks refer to tryptophan (370 nm), its metabolism products kynurenine and 3-hydroxykynurenine (439 and 470 nm), and carbamate anion (470 nm).

4.2 AN4 MicroTom plants genetic characterization by TLA and sequencing

Transgenic plants were obtained by *A. tumefaciens*-mediated transformation of MicroTom with the 35S:*PhAN4* construct. Plants harboring one copy of the *PhAN4* gene per haploid genome, were subjected to Targeted Locus Amplification (TLA) (De Vree et al., 2014). TLA was applied to confirm copy number and zygosity, to identify insertion sites and possible genetic alterations in the *PhAN4* transgene introduced upon transformation at the single nucleotide variants (SNVs) level. In particular, TLA was applied to the T₃ generation of the homozygous AN4-M and AN4-P₁ lines and of the AN4-P₂ hemizygous plants (chosen for the most favorable anthocyanin content, as it will be shown in the followings).

The Next Generation Sequencing (NGS) coverage obtained by TLA across the construct sequence in the AN4-M, AN4-P₁ and AN4-P₂ samples (Figure 4.11, Figure 4.12) across the construct sequence corresponding to the whole expression cassette within the LB and RB sequences allowed to proceed with the analysis (8,261-9,568 bp).

In total, three SNVs were found in the construct sequence, corresponding either to third position base changes not determining any amino-acid substitution, or to insertion in non-relevant positions (Table 4.2). In particular, the SNV arisen at the beginning of the *nptII* gene in position 8,261, corresponded to a third base Guanine changed into a Cytosine, leaving Leucine unchanged. The SNV identified in position 9,454 consisted of a Guanine insertion near to a Timine. This SNV was found in a not relevant region comprised between the 35S promoter and the *PhAN4* CDS. Lastly, the only SNV identified in the *PhAN4* CDS corresponded to a third position Guanine changed in an Adenine in position 9,568, leaving Arginine in position n.26 unchanged. TLA analysis allowed to determine the transgene (TG) integration sites (Table 4.3) (Figure 4.12). In AN4-P₁ (A) and in AN4-P₂ (B), *PhAN4* resulted to be integrated in chromosome 12: 23,824,716-23,824,827. In AN4-M (C), *PhAN4* resulted to be integrated in chromosome 8: 11,072,987-11,073,027.

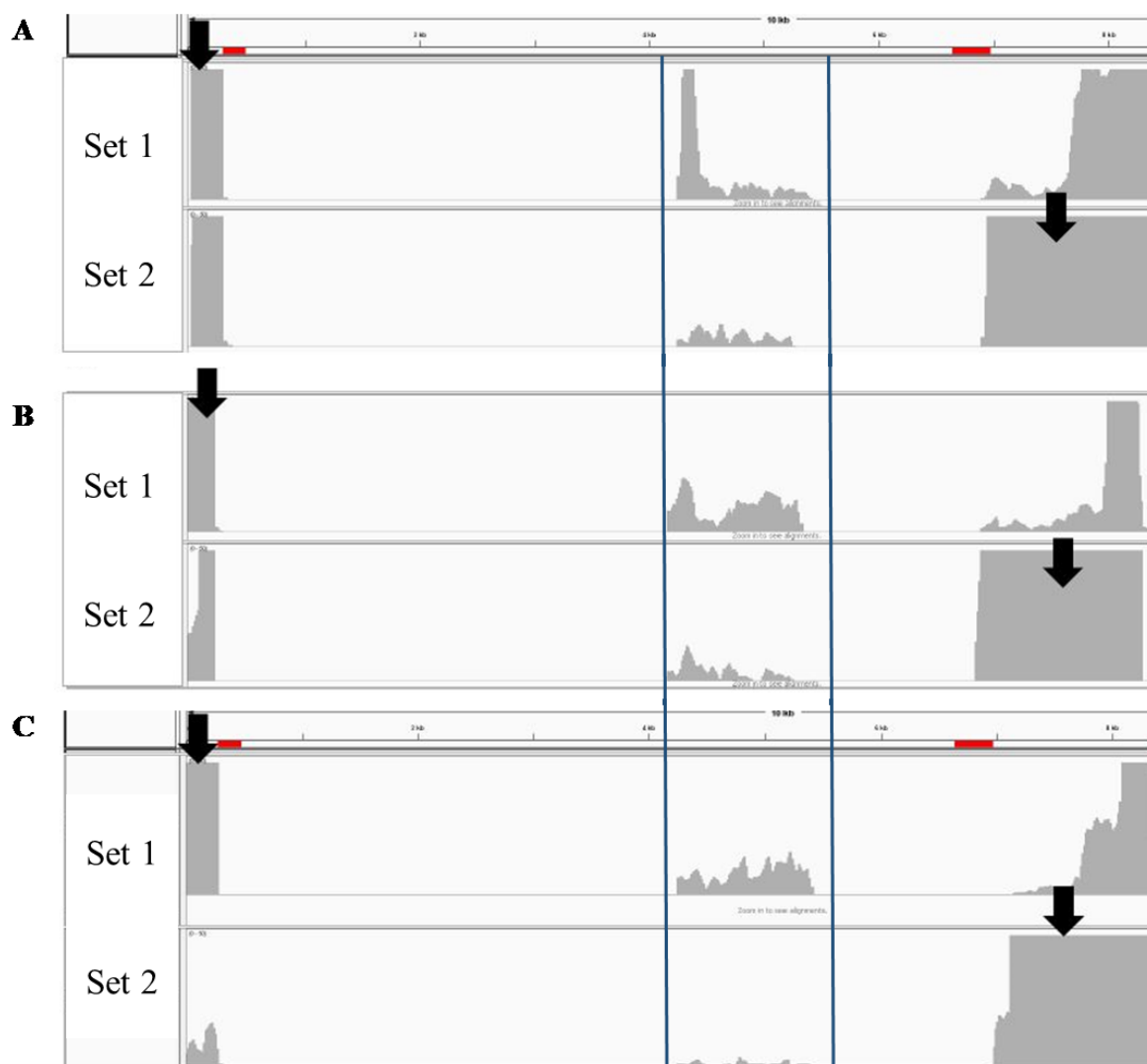


Figure 4. 11 NGS sequencing coverage across the construct of AN4-P₁ (A), AN4-P₂ (B) and AN4-M (C) samples. The sequence coverage was obtained with primer set 1 (top) and primer set 2 (bottom). Black arrows indicate primer location. RB and LB sequences are marked with red bars. The PhAN4 coding sequence is comprised between the two blue bars. Y-axis is limited to 50x.

Table 4.2 SNVs identified in AN4-P₁, AN4-P₂ and AN4-M samples. ‘+’ indicates an insertion.

AN4-P ₁ and AN4-P ₂			
Region	Position	Reference	Mutation
nptII	8261	G	C
backbone	9454	T	+1G
AN4 CDS	9568	G	A
AN4-M			
Region	Position	Reference	Mutation
nptII	8261	G	C
backbone	9454	T	+1G
AN4 CDS	9568	G	A

Table 4.3 Integration sites resulted from TLA analysis. The 3 SNVs refers to the upstream and downstream integration sites determining an interruption of the sequence in the same region of the untransformed control and to the SNV in the *PhAN4* CDS.

Sample	Integration site(s)	Notes
AN4-P ₁	12:23,824,716 – 23,824,827, homozygous sample	3SNVs
AN4-P ₂	12:23,824,716 – 23,824,827, also WT alleles present	3SNVs
AN4-M	8:11,072,987 – 11,073,027	3SNVs

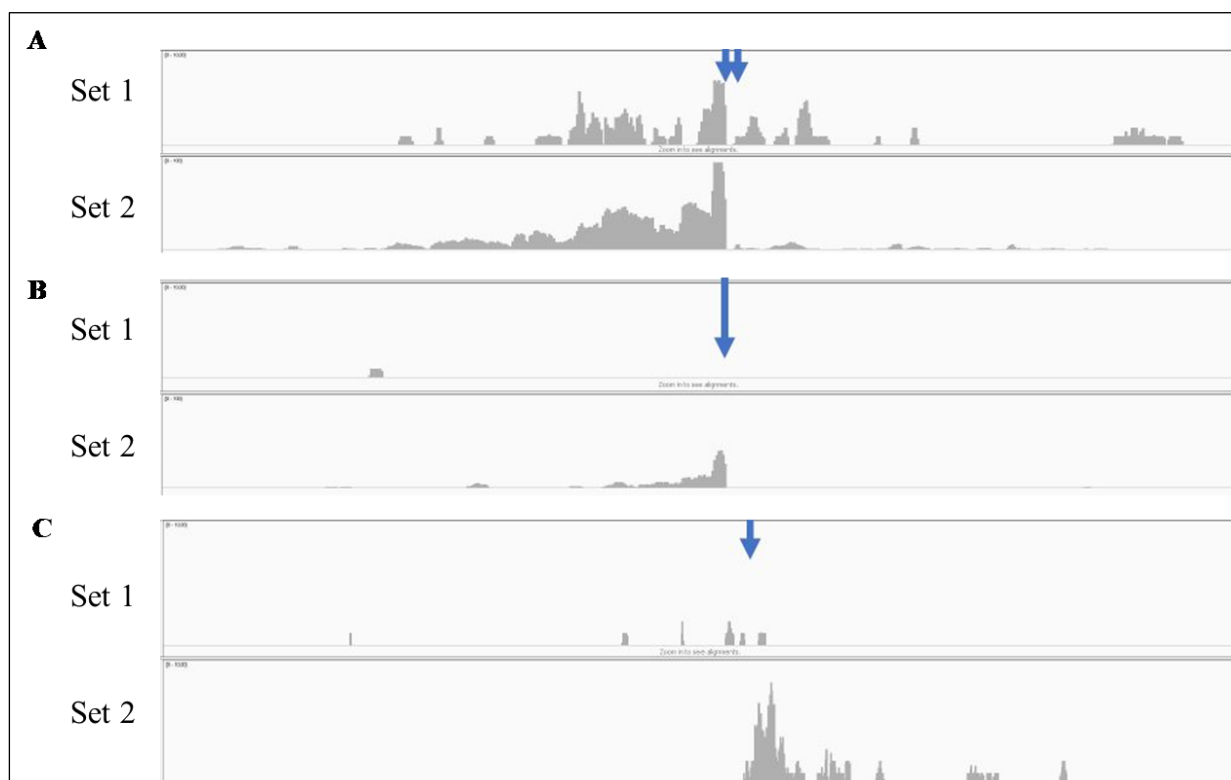


Figure 4.12 TLA sequence coverage across the TG integration locus; tomato chromosome 12: 23,824,716 – 23,824,827 in AN4-P₁ (A), AN4-P₂ (B) and chromosome 8: 11,072,987 – 11,073,027 in AN4-M (C). Sequence coverage (in grey) generated with primer set 1 (top) and primer set 2 (bottom). The blue arrows indicate the identified breakpoint sequences highlighting the integration sites. Y-axis is limited to 10x (set 1) and 100x (set 2).

The zygosity determination performed on samples AN4-P₁ and AN4-P₂ was accomplished using a primer set on chromosome 12 close to the identified integration site. No wild type reads were found between 12: 23,824,716 and 23,824,827 in AN4-P₁ sample, indicating that interruption of the chromosome 12 occurred due to the TG integration event. Conversely, in AN4-P₂, the presence of amplification reads for both the wild type and the TG, indicated the presence of a construct allele, beside a wild type allele (Table 4.4). Based on the obtained results, sample AN4-P₁ is homozygous for the TG allele while AN4-P₂ is hemizygous.

Table 4.4 Summary results obtained from the zygotity study conducted on AN4-P₁ and AN4-P₂ samples.

Sample	Border	Total number of reads	Number of fusion reads (%)	Number of WT reads (%)
AN4-P ₁	5'end	15	13 (87%)	0 (0%)
	3'end	2	2 (100%)	0 (0%)
AN4-P ₂	5'end	18	7 (39%)	11 (61%)
	3'end	5	1 (20%)	4 (80%)

In order to evaluate if the integration events either interrupted or relapsed into possibly relevant loci of the MicroTom genome, the corresponding regions of *Solanum lycopersicum* cultivar Heinz 1706 chromosome 12 SL3.0 (NC_015449.3) and chromosome 8 (NC_015445.3) (Figures 4.13 and 4.14) were analyzed. Not relevant coding regions were detected until ~ 600,000 bp upstream and downstream the integration sites within both chromosome 12 and 8, indicating that integration occurred without possibly disturbing known gene functions for none of the chosen transgenic plants.

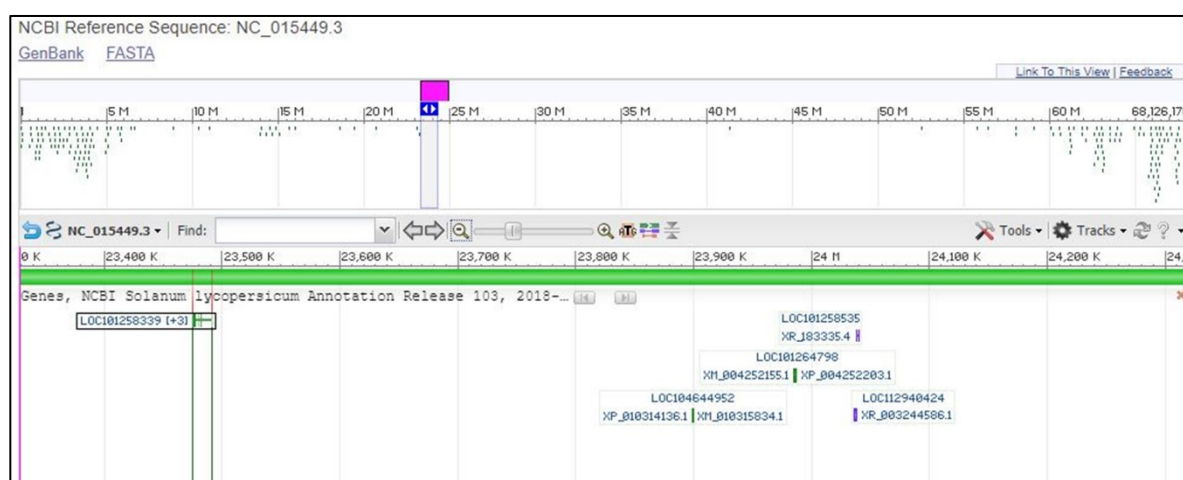


Figure 4. 13 Graphical visualization of the integration site of AN4-P₁ and AN4-P₂ samples. *Solanum lycopersicum* cultivar Heinz 1706 chromosome 12 SL3.0 (NC_015449.3) sequence was used.

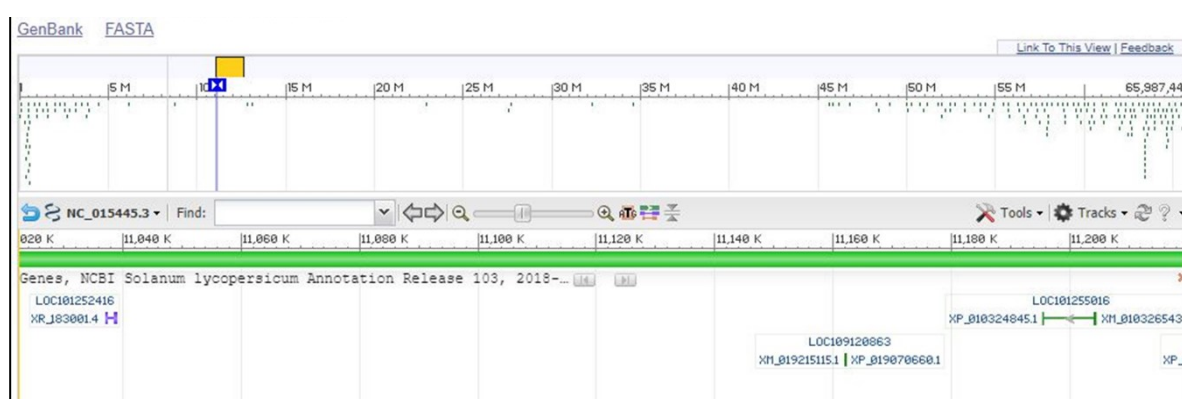


Figure 4. 14 Graphical visualization of the integration site of AN4-M sample. *Solanum lycopersicum* cultivar Heinz 1706 chromosome 8 SL3.0 (NC_015445.3) sequence was used.

4.3 Phenotypic characterization of MicroTom plants expressing *PhAN4*

The T₃ generation of AN4 MicroTom transgenic plants harboring one copy of the *PhAN4* gene on a single locus, were considered for subsequent phenotypic characterization. In particular, due to the most favourable features related to anthocyanins accumulation in fruits, the homozygous AN4-M line and the AN4-P₂ hemizygous plants were further characterized.

Despite with differences, both hemizygous T₃ AN4-P₂ and homozygous T₃ AN4-M plants, grown in hydroponic conditions in the ENEA BSL2 greenhouse, showed a purple pigmentation throughout their structure, from leaves and leaf veins, to stem and roots, to anthers and ovary. Purple pigmentation was particularly evident in the hemizygous AN4-P₂ in all developmental stages and structures from seed to whole plant compared to the AN4-M line (Figure 4.15), and this feature was maintained through several generations. Life-cycle traits, such as the duration of vegetative state, flowering time and senescence, were not significantly altered by the influence of the *PhAN4* gene. Indeed, in our cultivation conditions, the number of days from sowing to anthesis ranged from 37 to 44 days and the number of days from sowing to fruit development ranged from 65 to 72, for wild type and AN4 plants, respectively. These data fall within the ranges described for wild type MicroTom by Meissner et al., 1997.

	Flower	Ripening stages	Leaves	Roots	Whole plant
MicroTom wild type		 Immature green  Breaker  Breaker + 7 days  Red ripe			
<i>PhAN4-M</i> (homozygous, 'Magenta')		 Immature green  Breaker  Breaker + 7 days  Red ripe			
<i>PhAN4-P₂</i> (hemizygous, 'Purple')		 Immature green  Breaker  Breaker + 7 days  Red ripe			

Figure 4. 15 Plant parts phenotypes of T₃ generation MicroTom expressing PhAN4 gene under the control of the 35S promoter. Phenotypic analysis of wild-type, homozygous AN4-M line and hemizygous AN4-P₂ individuals. Tomato flowers, whole fruits (harvested at the immature green, breaker, breaker +7 days and red ripening stages) and related cross-section, leaves, roots and whole plants.

Conversely, vegetative traits resulted significantly different among wild type and engineered plants (Figure 4.16). Morphometric analysis, indeed, showed that both AN4-M and, more markedly, AN4-P₂ plants were characterized by a significantly smaller size than wild type due to a decreased height, number of leaves and decreased leaf area. On the other hand, yield traits such as number of flowers, number of fruits, diameter of fruits and weight of fruits, are not significantly affected by *PhAN4* ectopic expression, with the only exception of number of seeds that are significantly lower in AN4-P₂ plants compared to both wild type and AN4-M plants (Figure 4.16).

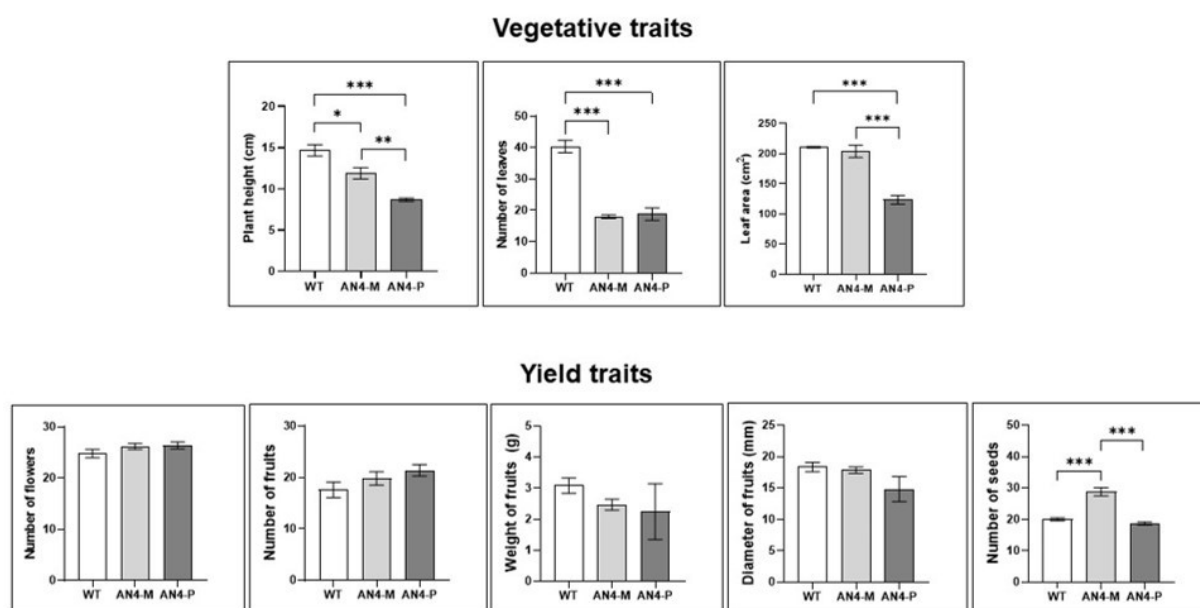


Figure 4. 16 Compared morphometric analysis among wild type, AN4-P2 and AN4-M plants. Means $\pm$ SE (n = 5 replicates).

Evaluations performed during plant development with Multiplex Force A™, showed that SFR and NBI indices progressively increased, as expected due to plant growth (and as shown by the trendline in Figure 4.17 and Figure 4.18). Along growth, a sort of periodic sigmoidal-like trend of values was observed for SFR and NBI for all the genotypes (mostly pronounced in wild type) but with different periodicity among wild type, AN4-M and AN4-P₂ plants. Especially for NBI, the anticipation of the first significant variation of the index is evident. In particular, AN4-M and AN4-P₂ transgenic plants anticipate by one week and two weeks, respectively, the first peak of NBI compared to wild type (37 DAS for P₂, 44 DAS for M and 51 DAS for wild type). NBI reflects the ratio between primary and secondary metabolism. This effect on the NBI index could be due to a decrease of the secondary metabolism level, as observed by visual inspection of transgenic plants, showing a sort of attenuation of general pigmentation in correspondence of these moments and in accordance with the FLAV index (Figure 4.19).

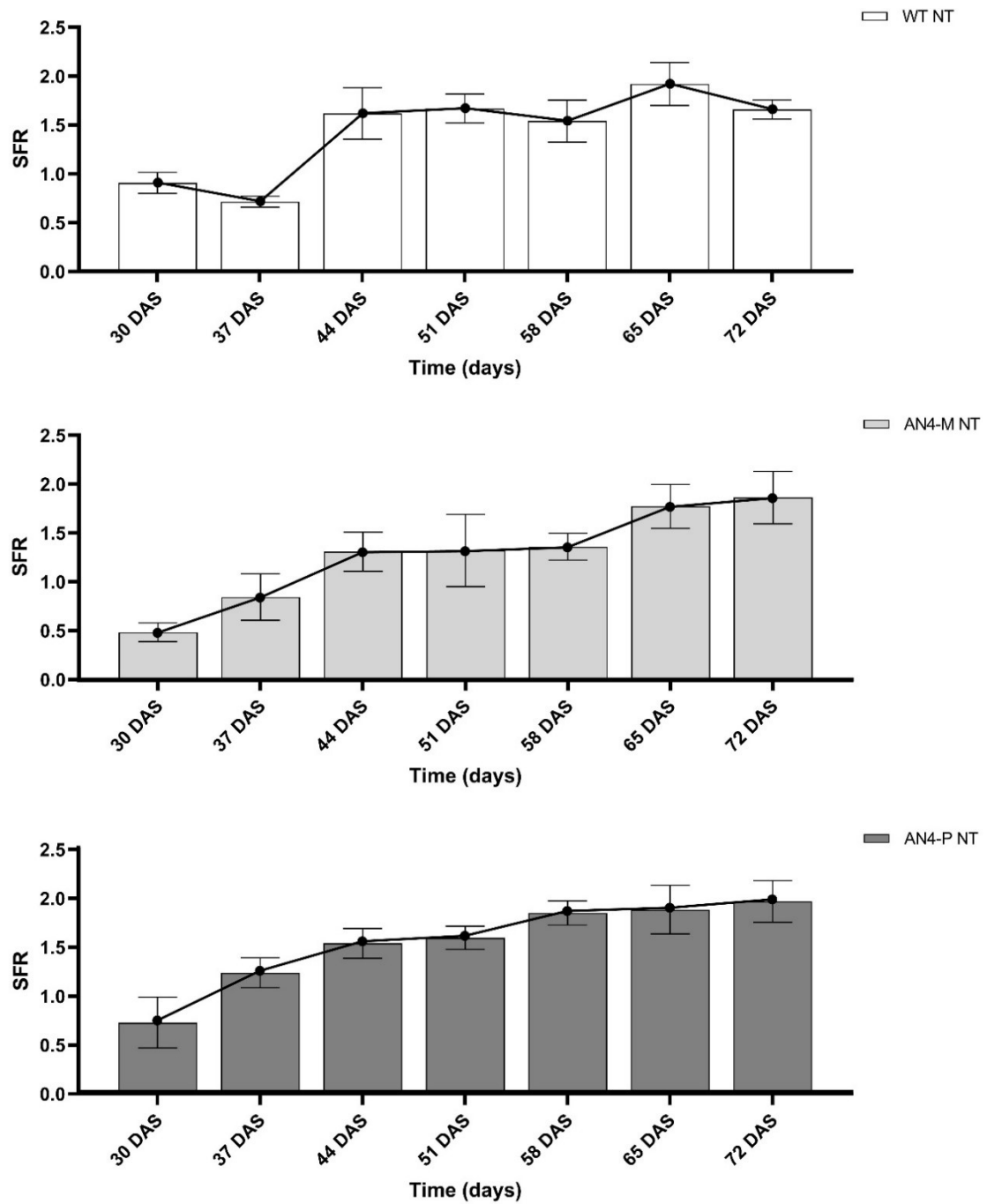


Figure 4. 17 Analysis of SFR index of wild type, AN4-M and AN4-P₂ MicroTom along the observation period.

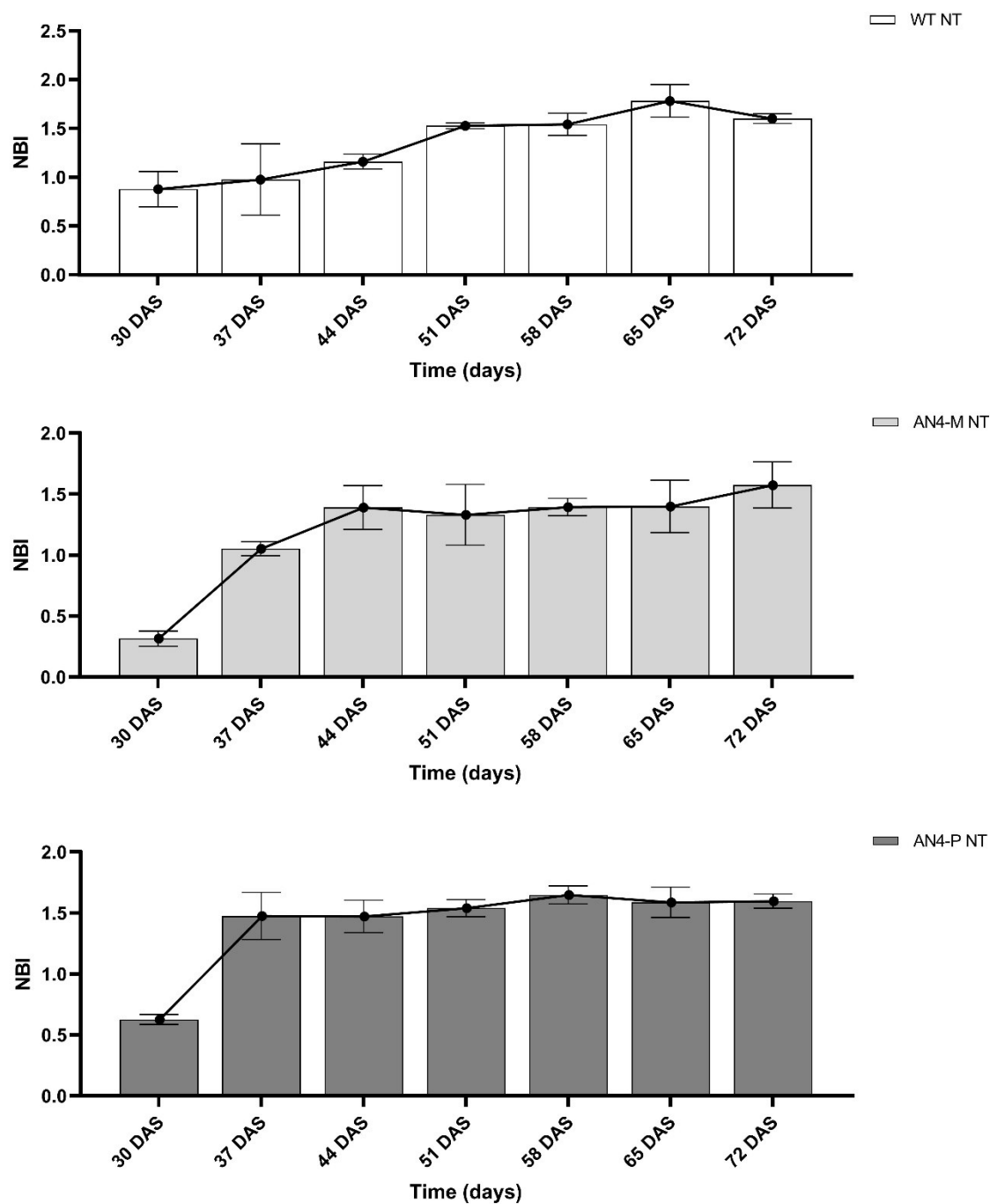


Figure 4. 18 Analysis of NBI index of wild type, AN4-M and AN4-P₂ MicroTom along the observation period.

The FLAV index values, as well, recorded a periodic sigmoidal-like shape trend for all the genotypes with several inflection points (Figure 4.19). The statistically relevant inversion of data towards negative values occurred at 51 days after sowing (DAS) in wild type plants, between 37 and 51 DAS in M plants, and between 30-37 DAS for P₂ plants. After this inversion point, FLAV values are generally higher for transgenic plants compared to wild type.

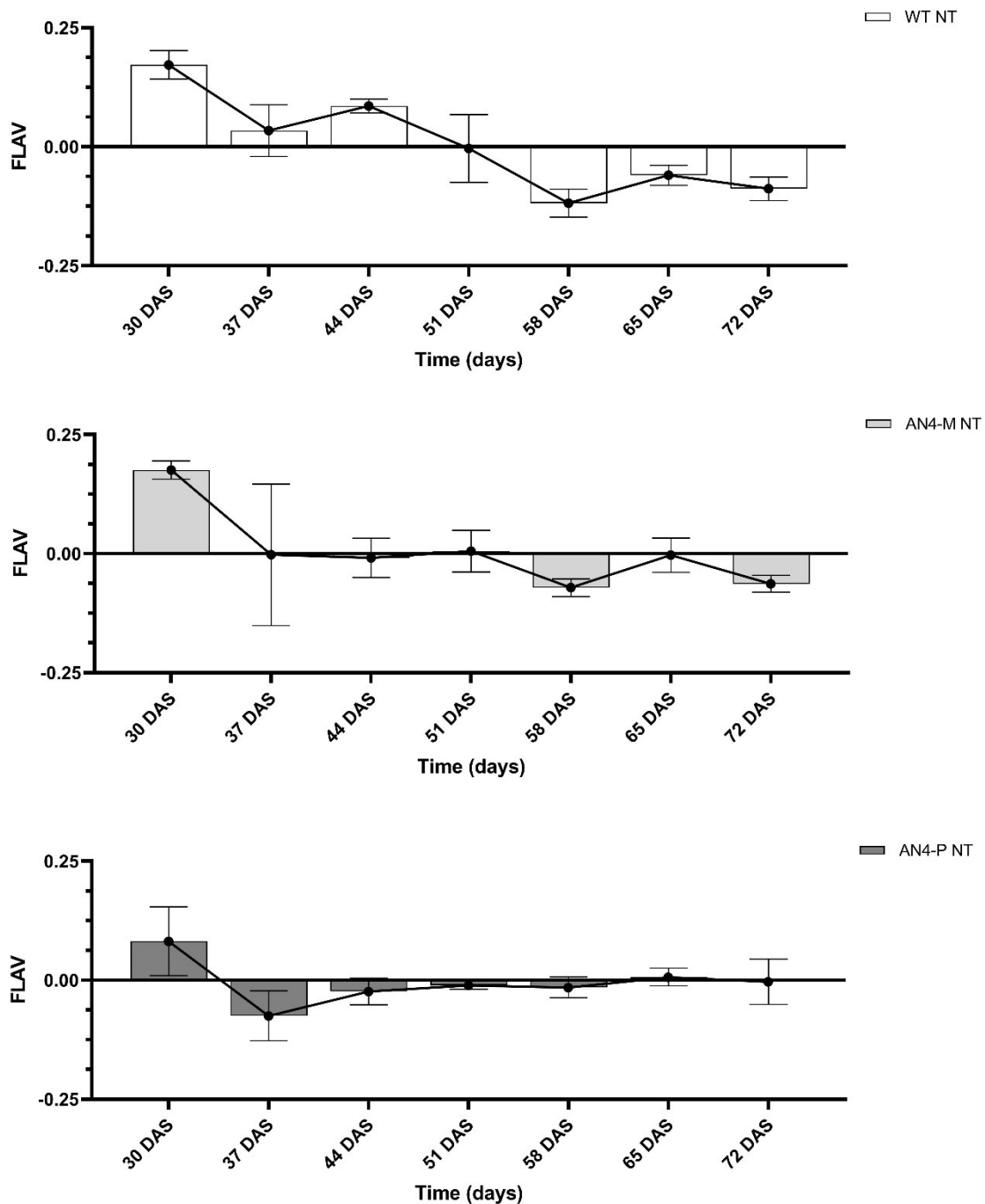


Figure 4. 19 Analysis of FLAV index of wild type, AN4-M and AN4-P₂ MicroTom along the observation period.

Interestingly, and as expected, the ANTH index values defined different curve shapes for the wild type compared to the two transgenic genotypes (Figure 4.20). Indeed, while ANTH values were constantly negative throughout life cycle of wild type, indicating absence of anthocyanins, AN4 plants were characterized by a sigmoidal curve with a statistically relevant inversion of values around 37-51 DAS, in accordance with the FLAV index.

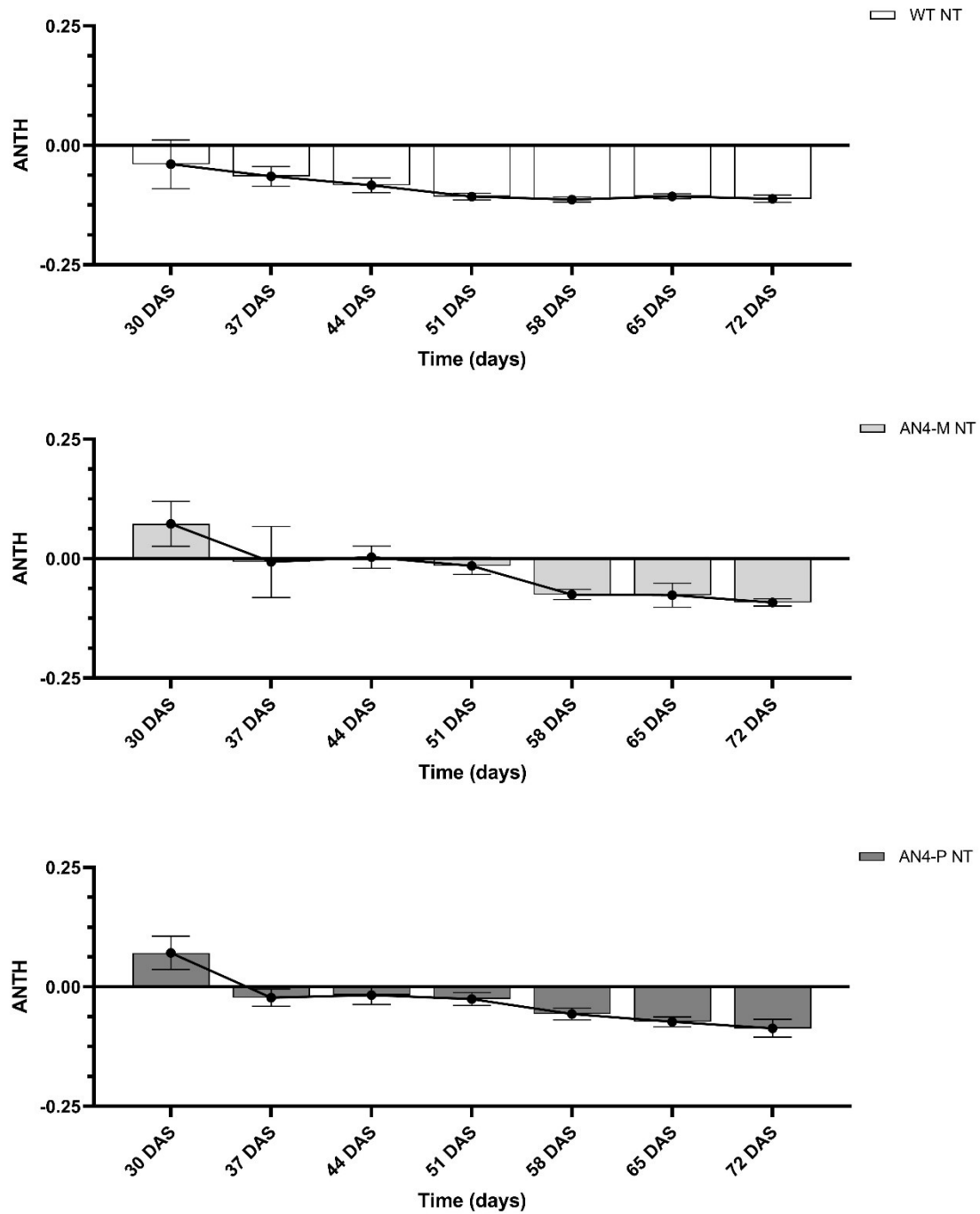


Figure 4. 20 Analysis of ANTH index of wild type, AN4-M and AN4-P₂ MicroTom along the observation period.

4.4 Anthocyanin content and antioxidant (DPPH) activity in wild type, AN4-M and AN4-P₂ fruits

Total anthocyanin content was measured in fruits along the ripening period (Figure 4.21). This was accomplished to evaluate the level of anthocyanins biosynthesis already found in the HRCs model, in transgenic AN4-M and AN4-P₂ fruits, as well.

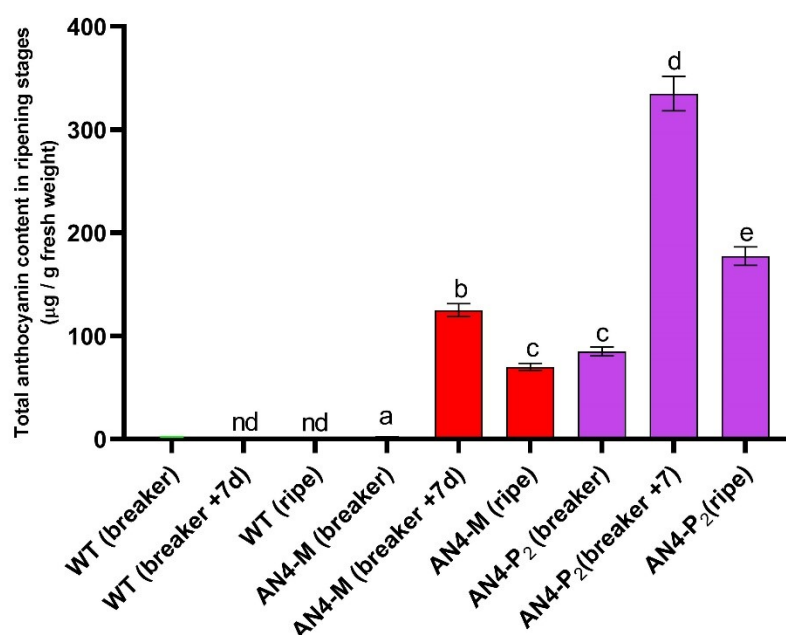


Figure 4. 21 Total anthocyanin content in wild type and engineered tomato fruits. The amount of anthocyanins is expressed in µg/g of fresh weight. Each analysis consisted of triplicate measurements of each sample and data were averaged over the three measurements. A univariate statistical analysis based on Student's test and one way-ANOVA ($P \leq 0.05$) was carried out.

In transgenic fruits, accumulation of anthocyanins (virtually absent in wild type), increased through the ripening stages until the breaker + 7d stage, when both AN4-M and AN4-P₂ plants showed the highest anthocyanins concentration, accumulating 125 ± 6 and 335 ± 17 µg/g of fresh weight, respectively. At complete ripening, the anthocyanin content decreased for both AN4-M (70 ± 4 µg/g fresh weight) and AN4-P₂ fruits (177.5 ± 8.9 µg/g fresh weight).

To verify whether the differential anthocyanin content upon *PhAN4* gene expression was associated to a variation in antioxidant activity, the DPPH assay was carried on (Figure 4.22). The DPPH antioxidant scavenging capacity of AN4-M fruits was statistically higher than wild type along the ripening stages with the only exception of the red ripe stage. The antioxidant activity of AN4-P₂ fruits was equal or significantly higher to wild time along all the ripening stages.

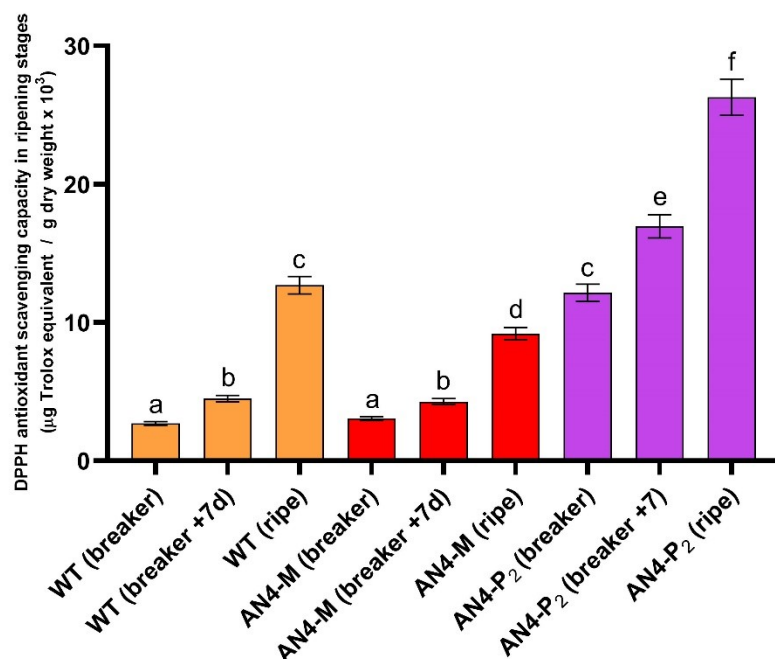


Figure 4. 22 Analysis of antioxidant activity (DPPH) in tomato fruits from wild type and PhAN4 transgenic plants. Results are expressed as μg Trolox equivalents / g dry weight (DW). Each analysis consisted of triplicate measurements of each sample and data were averaged over the three measurements. A univariate statistical analysis based on one way-ANOVA ($P \leq 0.05$) was carried out.

4.5 Characterization of anti-oxidant properties of wild type, AN4-M and AN4-P₂ fruits

High dose gamma radiation, being known to efficiently generate peroxy radicals, was initially used simply as an available tool to induce the formation of these chemical species in ripe fruits from wild type and transgenic MicroTom. In order to investigate how the differential metabolite content of ripe fruits may have different capacity to counteract the generation of ROS by gamma irradiation and to preserve plant protein folding, ESR and photoluminescence analysis were used, respectively. This research was carried on to evaluate the potential of *PhAN4*-biofortification to protect humans (as the basis of anti-oxidant space food) and plants themselves from oxidative damage.

4.5.1 Analysis of radical scavenging ability of wild type, AN4-M and AN4-P₂ fruits by ESR analysis

The ESR allowed to assess the amount of free radicals accumulated in the lyophilized biomass of transgenic fruits upon gamma irradiation compared to wild type (Figure 4.23). In general, all

the MicroTom irradiated samples independently from genotype, exhibited a composite ESR spectrum, due to the complexity of the biological matrix of the tomato fruit. The singlet of the peroxy radical ($g = 2.014$) could be easily identified (Bacić et al., 1993; Morsy and Khaled, 2002; Faure et al., 2014), consistently with the $^{\circ}\text{OH}$ -induced oxidation of carbohydrates, which includes the formation of peroxy radical intermediates. In each genotype, irradiation produced two side peaks (doublet) at about $\pm 3\text{mT}$ from each other ($g = 2.014$ and $g = 2.020$), whose hyperfine constants were found to be the same as that of irradiated cellulose. Therefore, these peaks are attributable to the radiation-induced cellulose radicals (Raffi and Agnel, 1989; Raffi et al., 2000; Delincée and Soika, 2002; Aleksieva et al., 2009; Yordanov and Aleksieva, 2009; Ahn et al., 2013; Akram et al., 2013; Dului and Bercu, 2017).

The ESR maximum intensity of radicals accumulation after irradiation showed a significant lower intensity of the peroxy radical singlet ($g = 2.020$) in AN4-M compared to wild type ($\Delta h = -470.16$) (Figure 4.23A), whereas radicals referring to the $g = 2.014$ singlet accumulated at a slightly higher level in AN4-M ($\Delta h = 162.46$). The ESR spectra and the maximum intensity (h) reached by radicals accumulation for AN4-P₂ and wild type (Figure 4.23B) are substantially equivalent in correspondence of radical referring to the $g = 2.020$ ($\Delta h = -232.96$), whereas they differ for the $g = 2.014$ singlet with AN4-P₂ showing a significantly lower level of radicals accumulated after irradiation compared to wild type ($\Delta h = 20.57$). The value calculated for the overall area under the curve (a.u.c.) defined by the doublet $g = 2.014 / g = 2.020$ is similar in AN4-M and AN4-P₂ (a.u.c._{AN4-M} = 7,717; a.u.c._{AN4-P} = 7,716), whereas is lower with respect to wild type (a.u.c._{wt} = 9,717). This means that, by exposure to an equal dose of radiation, AN4-P₂ and AN4-M ripe fruits metabolite content have a significantly higher radical scavenging ability compared to wild type, resulting in a significantly overall lower level of radicals accumulated.

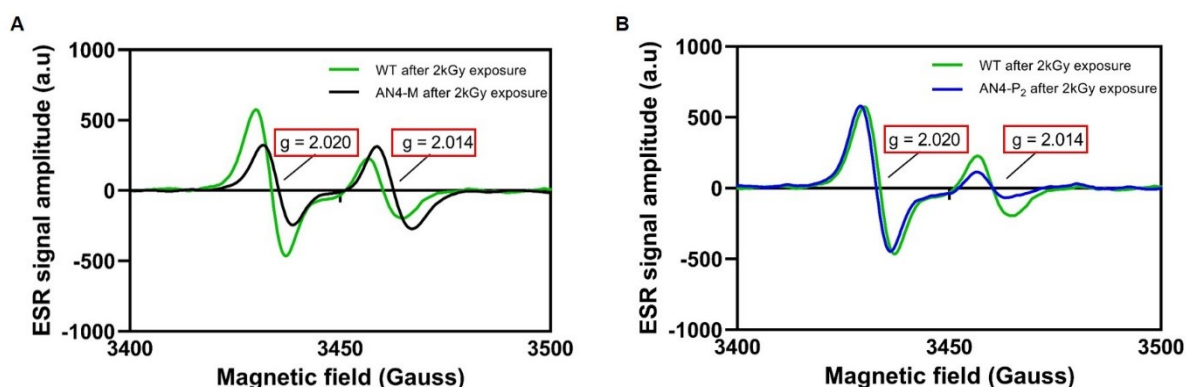


Figure 4. 23 ESR spectra of wild type, AN4-M (A) and AN4-P fruits (B) at 2 kGy absorbed dose (dose rate = 1.8kGy/h). The intensity of each signal is expressed as the peak-to-peak height normalized for mass unit and by subtracting the intensities of the signals before irradiation.

4.5.2 Analysis of oxidation of wild type, AN4-M and AN4-P₂ fruit soluble proteins by photoluminescence

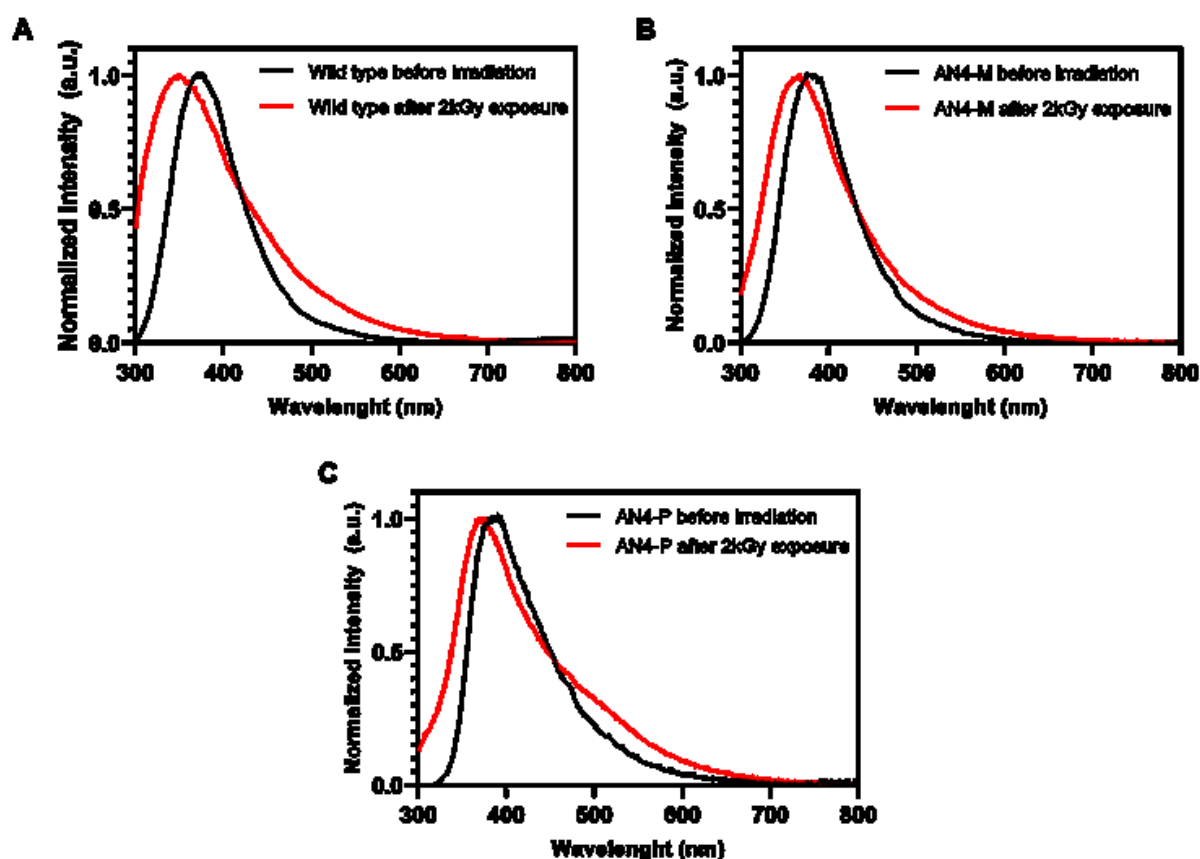
To evaluate the resistance to misfolding and to oxidation of MicroTom fruit soluble proteins may be influenced by the differential metabolic content upon a potent pro-oxidant stimulus, the photoluminescence (PL) spectra of wild type and AN4-M and AN4-P₂ ripe fruits were evaluated upon high dose gamma irradiation (Figure 4.24). When exposed to the wavelength of 280 nm, the phenylalanine, tryptophan and tyrosine residues of protein molecules are excited and the maximum emission peak position reflects upon secondary and tertiary structure.

Photoluminescence spectra of extracts containing soluble proteins from wild type, AN4-M and AN4-P₂ fruits were produced. The mass-normalized emission spectra of not irradiated soluble protein samples, are similar in wild type, AN4-M and AN4-P₂ and are characterized by a peak mainly corresponding to tryptophan (at 371 nm and 376 nm, respectively) (Yang et al., 2015a; Hilaire et al., 2017). Trp peak, that is known to be highly dependent on polarity and/or local environment, is shifted at 391 nm in AN4-P₂ extracts in our experimental conditions.

High dose gamma irradiation did not cause any detectable decrease in fluorescence emission intensity of any of the spectra, independently from genotype, demonstrating a general resistance of MicroTom fruit soluble proteins tertiary and quaternary structure to this stress.

Upon irradiation, a blue shift phenomenon was observed in the spectra of all the three genotypes, being more relevant in wild type (-21 nm) and AN4-M (-16 nm) than in AN4-P₂ (- 9 nm) and revealing that the side chain indole of Trp of proteins of the first two genotypes are more prone to undergo oxidation upon strong gamma radiation than AN4-P₂. As a consequence, in wild type and AN4-M a shoulder appears (306-310 nm) in the emission maximum mainly referring to Tyr.

In AN4-P₂ irradiated proteins, Trp derivatives such as kynurenine and 3-hydroxykynurenine (439 and 470 nm) are probably formed (Lohmann et al., 1988; Daly et al., 2009; Gakamsky et al., 2017). In addition, a peak at 470 nm is indicative of the presence of carbamate anions (Pan et al., 2013).



Sample	Peak Bef. irr. (nm)	Normalized Intensity Bef. irr. (a.u.)	Peak After irr. (nm)	Normalized Intensity After. irr. (a.u.)	Delta blue-shift (nm)
Wild type	371 nm	1.006338	350 nm	1.000000	-21 nm
AN4-P	381 nm	0.99821	375 nm	0.997528	- 16 nm
AN4-M	376 nm	1.004632	367 nm	0.997555	- 9 nm

Figure 4. 24 Photoluminescence emission spectra in normal conditions and after 2 kGy exposure of wild type (A), AN4-M (B) and AN4-P₂ (C).

4.6 Effects of the gamma component of cosmic radiation on AN4 MicroTom seeds and plants simulating different scenarios on Mars

The effects of three different absorbed doses of gamma rays, as a simulated component of cosmic radiation related to Mars mission, were evaluated. Irradiation experiments were conducted on both wild type and engineered MicroTom dry seeds and on two sets of plants at two developmental stages (i.e. 30 and 45 days after sowing, DAS). In addition, to investigate the outcome of parental irradiation on related first-generation descendants, analyses were conducted on offspring obtained from plants irradiated at 30 DAS, as well.

As already described, biometric measurements in absence of radiation challenge (i.e. upon controlled BSL2 greenhouse conditions), had already revealed that the engineered genotypes AN4-M and AN4-P₂ are significantly different from wild type by general smaller size (e.g. decreased height, leaf area, number of leaves) despite comparable in terms of yield traits such as number of flowers, number of fruits, diameter of fruits and weight of fruits (with the only exception of number of seeds that are significantly higher for AN4-M plants). For this reason, plants had to be necessarily analysed by comparison within the respective genotype, after each radiation challenge treatment.

4.6.1 Biometric and fluorimetric characterization of plants generated by irradiated seeds

Equal amounts of wild type, AN4-M and AN4-P₂ seeds (80) were sub-grouped and irradiated with 0 (control), 0.5, 5 and 30 Gy absorbed doses of ⁶⁰Co gamma radiation. Both controls and treated seeds were then placed into *Petri* dishes on filter paper disks and soaked in the dark at 20°C. The percentage of plantlets (PP) risen thereof was calculated at 15 DAS. As expected from literature, the exposure of seeds to gamma radiation caused a slight decrease (5-10%) of PP compared to controls in wild type (5 Gy), AN4-M (0.5 and 5 Gy), and in AN4-P₂ (5 Gy). In some cases, PP remained either unchanged (wild type at 30 Gy, AN4-M at 30 Gy) or resulted to be increased (wild type at 0.5 Gy, AN4-P₂ at 0.5 Gy and 30 Gy) compared to controls (0 Gy). In any case, at 15 DAS, more than 70% of irradiated seeds had produced a plantlet, irrespective of genotype and irradiation dose (Table 4.5).

In order to simplify the manipulation of the data deriving from the detection of the chosen parameters, and to study the possible association between genotypes, investigated traits and irradiation treatments, a PCA (Principal Component) analysis was performed on data obtained from plants germinated from irradiated seeds (Figure 4.25).

Table 4.5. Plantlets percentage at 15 DAS.

Plantlets percentage at 15 DAS	0 Gy	0.5 Gy	5 Gy	30 Gy
wild type	75%	80%	70%	75%
AN4-M	80%	70%	75%	80%
AN4-P ₂	72.5%	75%	70%	77.5%

The PCA analysis was performed on the dataset obtained from seeds irradiated at 0.5 and 5 Gy due to the presence of a few outliers in the 30 Gy data matrix. Indeed, since the PCA analysis only includes rows that contain variables, when the value for any variable on that row is empty (missing) or excluded (outlier), rows are skipped.

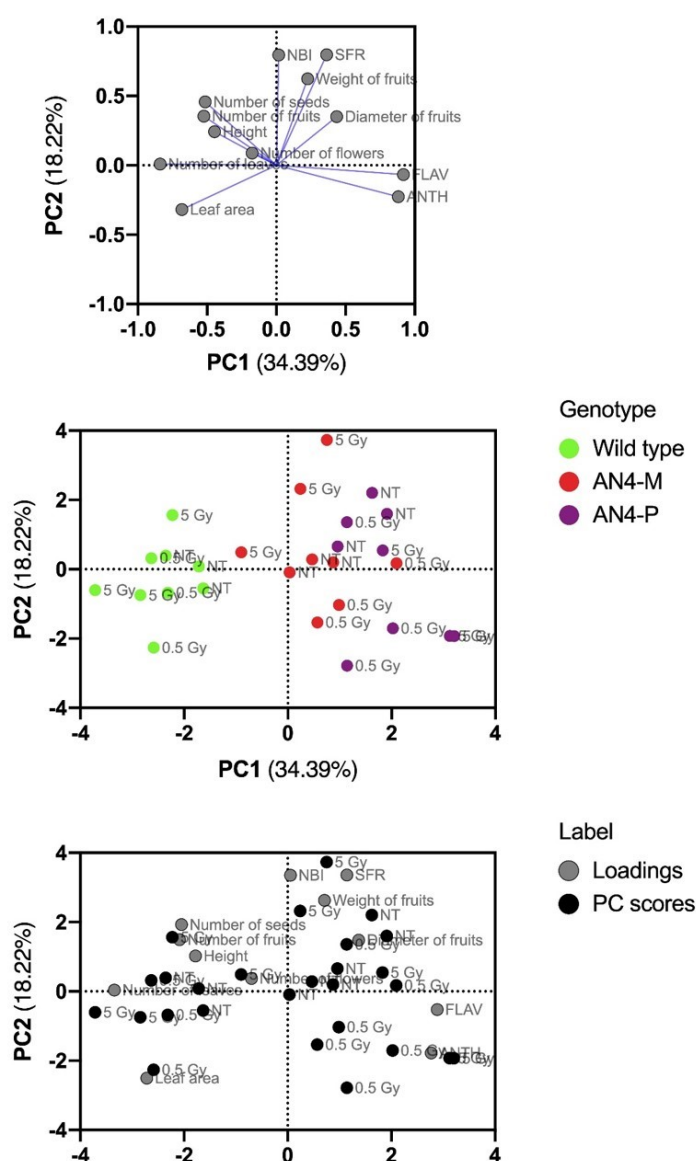


Figure 4. 25 Principal component analysis on morphometric and fluorimetric parameters obtained from measurements of plants germinated from irradiated seeds. Results are graphically reported using the Loadings plot with all the parameters considered (A), the PC scores plot with wild type and AN4 plants (B) and the biplot that contains the information presented in both the Loadings plot and the PC scores plot at the same time (C).

The contributions of the first two PCs were 34.39% (PC1) and 18.22% (PC2), respectively, and their sum explained the 52.61% of the phenotypic variation. Since the loadings for a principal component represent the correlation between the variables and the principal components, the Loadings plot (Figure 4.25A) shows that FLAV and ANTH variations are positively correlated with the PC1. This means that PC1 (i.e., the PC weighting the most on overall phenotypic variation) is mostly positively dominated by FLAV and ANTH and, therefore, that the main variation among the tested genotypes is mostly due to differences in these index levels among genotypes, even though variation can be due also to SFR, diameter and weight of fruits. Conversely, PC1 is negatively correlated to leaf area, number of leaves, plant height and number of fruits and seeds.

NBI, SFR, number, diameter and weight of fruits and number of seeds positively dominate PC2, while leaf area, FLAV and ANTH negatively dominate PC2. SFR, weight and diameter of fruits are positively correlated with both PC1 and PC2. Leaf area is negatively correlated with both PC1 and PC2.

The score plot and the biplot (Figure 4.25B and Figure 4.25C) allowed to differentially cluster the wild type with respect to the transgenic plants, facilitating the classification of their phenotypic variations. Indeed, while wild type plants (deriving from both irradiated and not-irradiated seeds) clustered in the left quadrants (PC1 negative values, positive or negative variations of PC2), plants deriving from both AN4-M and AN4-P₂ seeds, clustered mostly in the right quadrants of the plots (PC1 positive values, positive or negative variations of PC2), independently from radiation dose and genotype. This result shows that, as in part expected due to the *PhAN4* transgenesis, variations among plants observed upon radiation experiment can be mainly ascribed to the differential accumulation of the metabolites influencing the indices that are positively correlated to PC1, FLAV and ANTH, than to radiation.

The position of the AN4-M plants deriving from non-treated seeds, close to the origin of the axes of the score plot and the biplot, shows their limited contribution to the overall variation observed along the experiment. Interestingly, while the AN4-M plants deriving from 5 Gy-treated seeds together with the plants deriving from AN4-P₂ seeds irradiated with any dose or not irradiated clustered in the upper-right part of the plot (positive variations of both PC1 and of PC2, dominated by all the parameters with the only exception of leaf area), the plants deriving from the 0.5 Gy-treated AN4-M seeds mainly localized in its lower-right part (positive variation of PC1 but negative for PC2, dominated by NBI, SFR, weight and diameter of fruits, FLAV, ANTH and leaf area) together with the plants deriving from the 0.5 Gy-treated AN4- P₂ seeds.

The analysis of variance (two-way Anova) performed on data obtained at the end of the detection period (i.e., fruit set), revealed that the variables ‘time’ and ‘gamma irradiation’ are independent. Indeed, their interaction did not exceed the 4.61% of the total variation, demonstrating that the effect of gamma irradiation does not depend on the effect of the other independent variable time and viceversa. The percentage of total variation ascribed to the ‘gamma irradiation’ variable resulted between 0.002% and 2.31%. Conversely, the percentage of variation ascribed to the ‘time’ variable ranged between 72.7% and 94.7%, revealing that the variations observed upon the experiment are mostly due to the variable ‘time’, and that the variable ‘gamma irradiation’ only partially influence morphometric parameters of plants obtained from irradiated seeds. Indeed, considering the values recorded at the end of the detection period, irradiation of dry seeds did not reveal significant effects on plants biometry when compared to respective non-irradiated controls, with the only exception of parameters inherent fruits (summarized in Figure 4.26). Irradiation of seeds influenced the deriving plants of all the three genotypes, with positive effects on fruits of wild type and AN4-M genotypes (starting from 5 Gy adsorbed dose) and adverse effects on fruits of AN4-P₂ plants (starting from 0.5 Gy). In accordance with PCA, significantly positive variations of the number of seeds and of diameter of fruits were recorded for wild type plants deriving from seeds irradiated with 5 and 30 Gy, respectively (Figure 4.27 and Figure 4.28). In AN4-M plants, significant positive variations of the number of seeds and of the weight of fruits were recorded after irradiation of seeds with 5 and 30 Gy, respectively (Figure 4.28 and Figure 4.29). In AN4-P₂ plants, negative variations of weight of fruits (after 0.5 and 5 Gy irradiation of seeds), diameter of fruits (after 0.5 Gy irradiation of seeds) and number of seeds (after 0.5, 5 and 30 Gy irradiation of seeds) were recorded (Figure 4.27, Figure 4.28 and Figure 4.29).

	WT NT	WT 0.5Gy	WT 5Gy	WT 30Gy	
Height (cm)	9.37±0.23	8.87±0.42	8±0.4	9.5±0.28	+ ***
Number of leaves	28.75±0.75	29±1	29.75±0.25	29.75±0.25	+ **
Leaf area (mm ²)	202.54±19.01	199.67±5.47	214.07±6.16	196.48±5.24	+ *
Number of flowers	9.5±2.9	6.5±1.55	14.5±6.39	9.25±3.9	/
Number of fruits	21.25±4.21	23.5±3.94	18.5±3.37	22.5±1.44	- *
Weight of fruits (g)	2.51±0.2	2.47±0.2	3.1±0.2	3.3±0.35	- **
Diameter of fruits (mm)	13.66±0.46	12.6±0.47	14.86±0.66	15.06±0.88	- ***
Number of seeds	24.6±1.77	28±2.04	32.66±2.78	21±1.7	

	AN4-M NT	AN4-M 0.5Gy	AN4-M 5Gy	AN4-M 30Gy
Height (cm)	8.5±0.28	8.25±0.49	8.75±0.47	8±0.4
Number of leaves	23.25±0.47	22.5±0.64	22.75±0.75	22.5±0.64
Leaf area (mm ²)	152.16±20.07	156.97±14.06	156.48±15.93	171.65±5.24
Number of flowers	3.5±1.25	2.25±1.31	2±1.22	7.5±1.25
Number of fruits	14.75±1.49	14.5±2.1	20±2.04	17.5±0.95
Weight of fruits (g)	2.18±0.21	1.98±0.21	2.94±0.27	3.1±0.22
Diameter of fruits (mm)	12.13±0.6	12.66±0.82	13.93±0.86	16.26±1.78
Number of seeds	20±1.65	20±1.49	28±1.89	32.66±2.16

	AN4-P NT	AN4-P 0.5Gy	AN4-P 5Gy	AN4-P 30Gy
Height (cm)	8.25±0.74	8.25±0.54	8.25±0.67	8.5±0.64
Number of leaves	18±1.22	20.25±0.85	17.5±1.7	19±1.29
Leaf area (mm ²)	163.26±17.52	171.77±9.54	168.45±10.07	177.85±13.14
Number of flowers	4.25±1.25	7.75±1.65	7.75±1.03	7.25±1.03
Number of fruits	18.5±1.7	14.75±4.49	12.75±1.31	17.5±2.59
Weight of fruits (g)	3.44±0.22	1.99±0.2	2.31±0.24	2.68±0.27
Diameter of fruits (mm)	15.8±0.48	12±0.62	14.2±0.88	14.13±0.9
Number of seeds	32.66±2.18	17.33±1.48	20±1.67	20±1.38

Figure 4. 26 Summary of morphometric results at the end of the measurement period analysed in comparison within each genotypes and according to the different treatments on irradiated seeds. Data were subjected to two-way analysis of variance (two-way ANOVA) with Tukey's post-hoc test to determine the differences in average of all tested parameters between irradiated and non-irradiated plants. NT: non-treated.

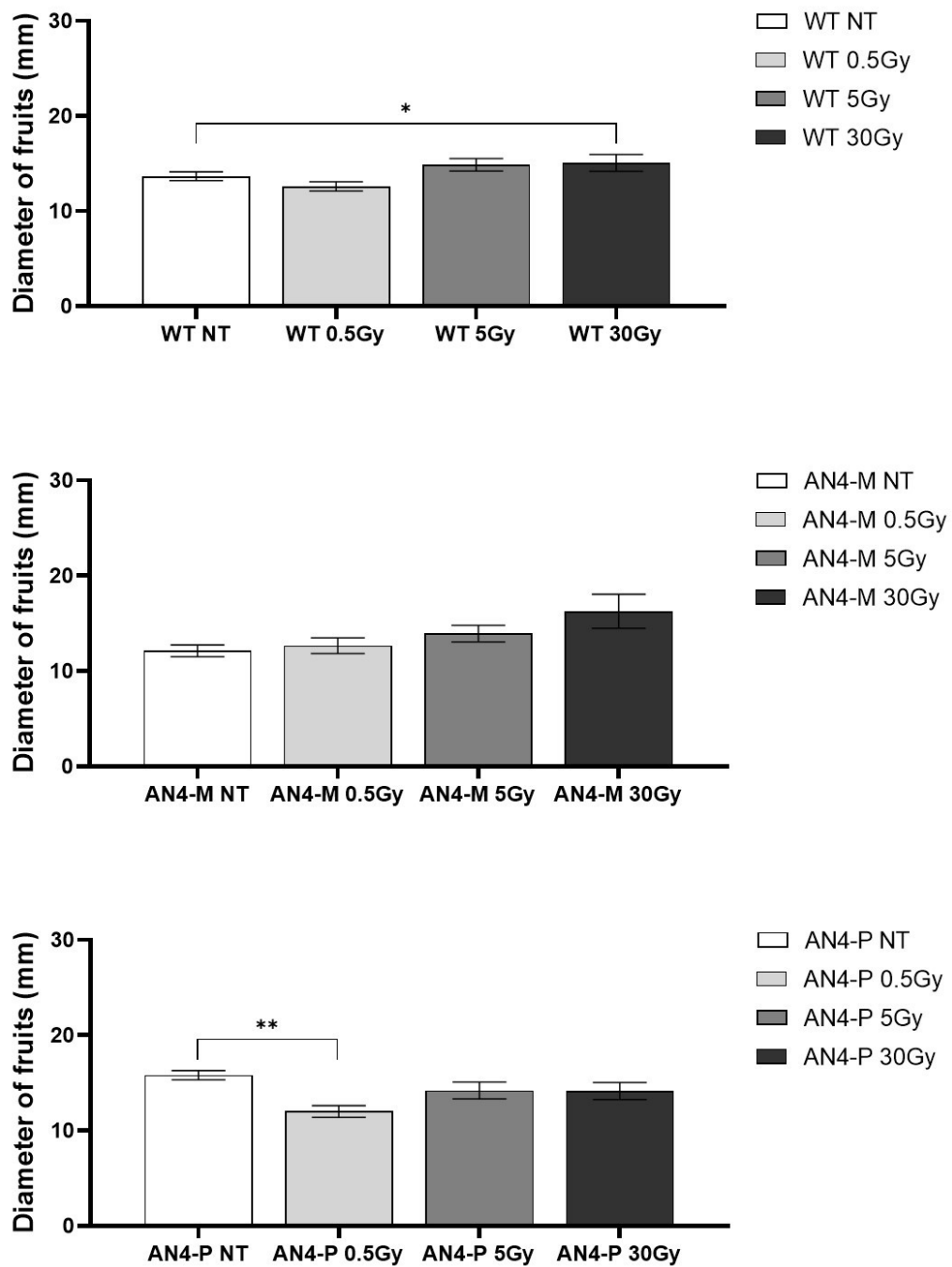


Figure 4. 27 Analysis of diameter of ripe fruits of wild type, AN4-M and AN4-P₂ MicroTom plants at the end of the life cycle upon the different irradiation treatments. Statistical relevance: *: $p < 0.05$; **: $p < 0.001$; ***: $p < 0.0001$.

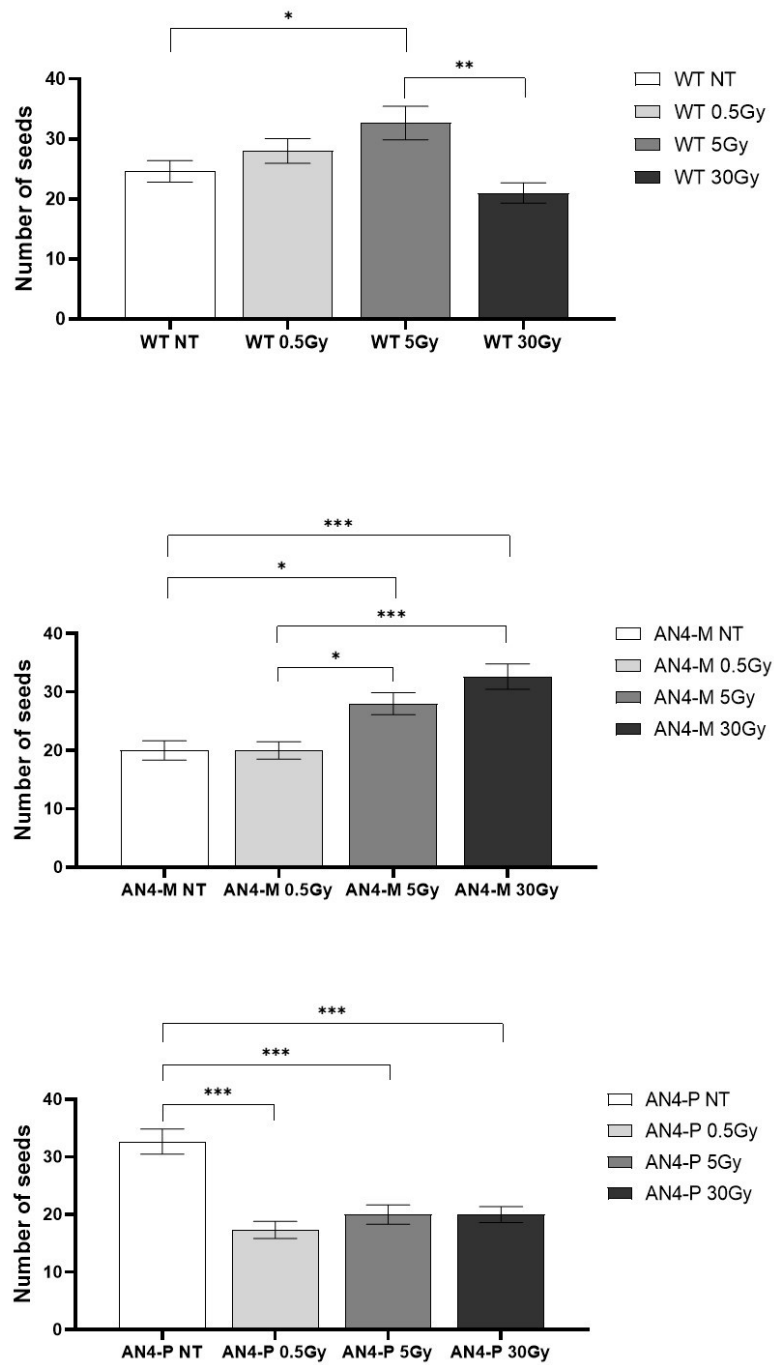


Figure 4. 28 Analysis of number of seeds of wild type, AN4-M and AN4-P₂ MicroTom plants at the end of the life cycle upon the different irradiation treatments. Statistical relevance: *: $p < 0.05$; **: $p < 0.001$; ***: $p < 0.0001$.

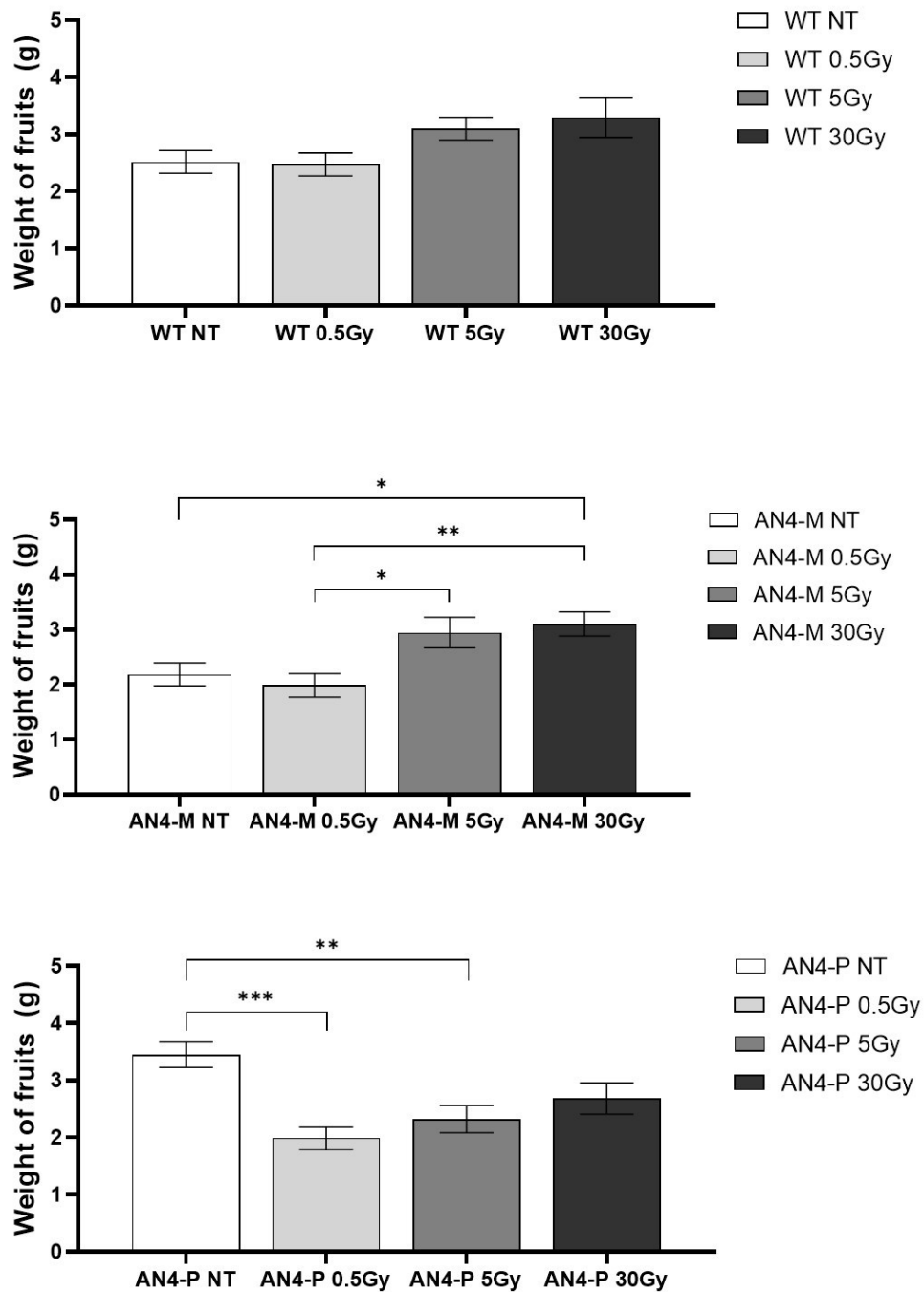


Figure 4. 29 Analysis of weight of ripe fruits of wild type, AN4-M and AN4-P₂ MicroTom plants at the end of the life cycle upon the different irradiation treatments. Statistical relevance: *: $p < 0.05$; **: $p < 0.001$; ***: $p < 0.0001$.

Throughout the measurement period, the analysis did not reveal statistically relevant differences in height, number of leaves, leaf area, number of flowers and number of fruits in plants obtained from irradiated seeds, in function of the adsorbed dose within each genotype (from Figure 4.30 to Figure 4.34).

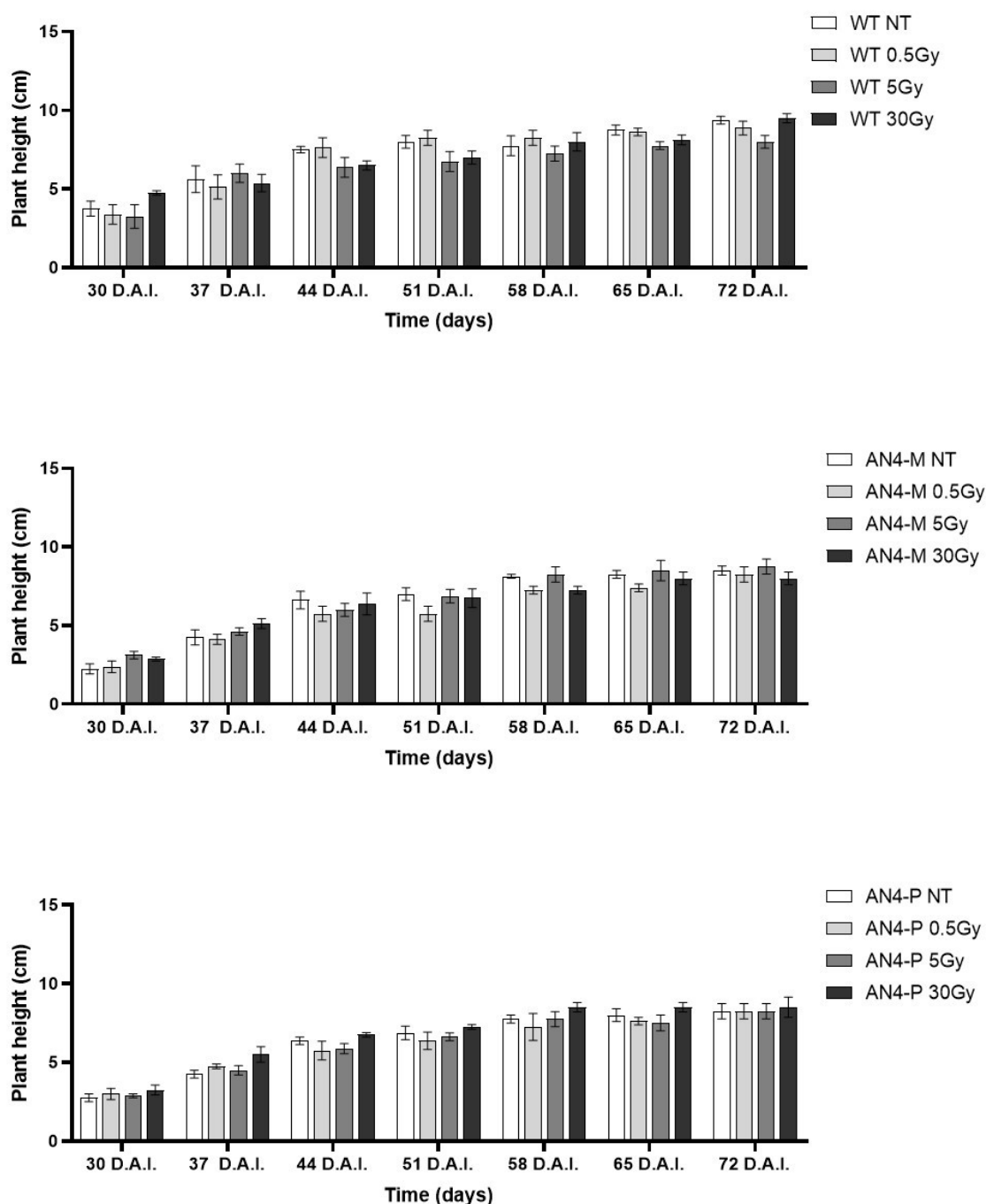


Figure 4. 30 Analysis of plant height of wild type, AN4-M and AN4-P₂ MicroTom along the observation period upon different irradiation treatments. Statistical relevance: *: $p < 0.05$; **: $p < 0.001$; ***: $p < 0.0001$.

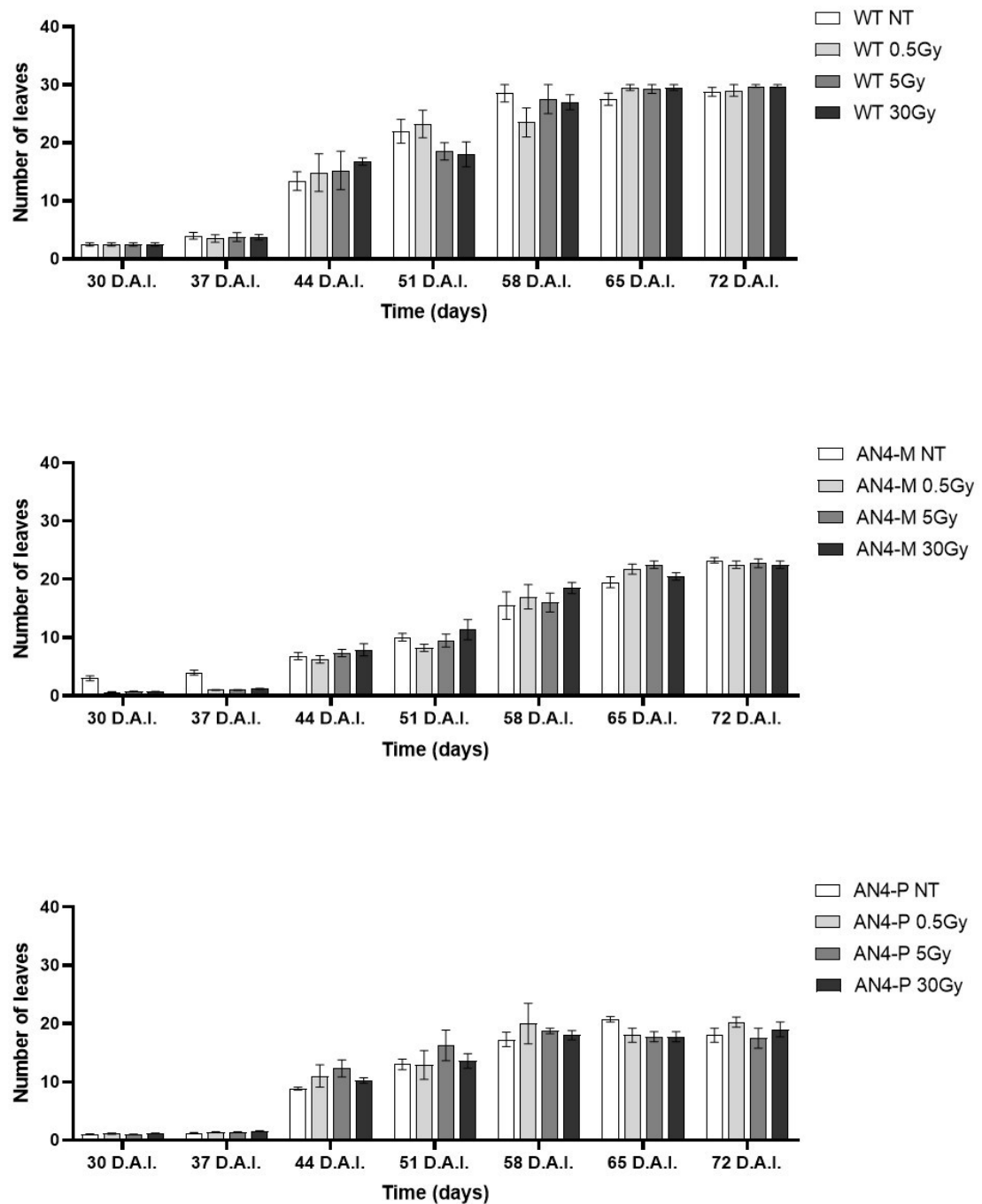


Figure 4. 31 Analysis of number of leaves of wild type, AN4-M and AN4-P₂ MicroTom along the observation period upon different irradiation treatments. Statistical relevance: *: $p < 0.05$; **: $p < 0.001$; ***: $p < 0.0001$

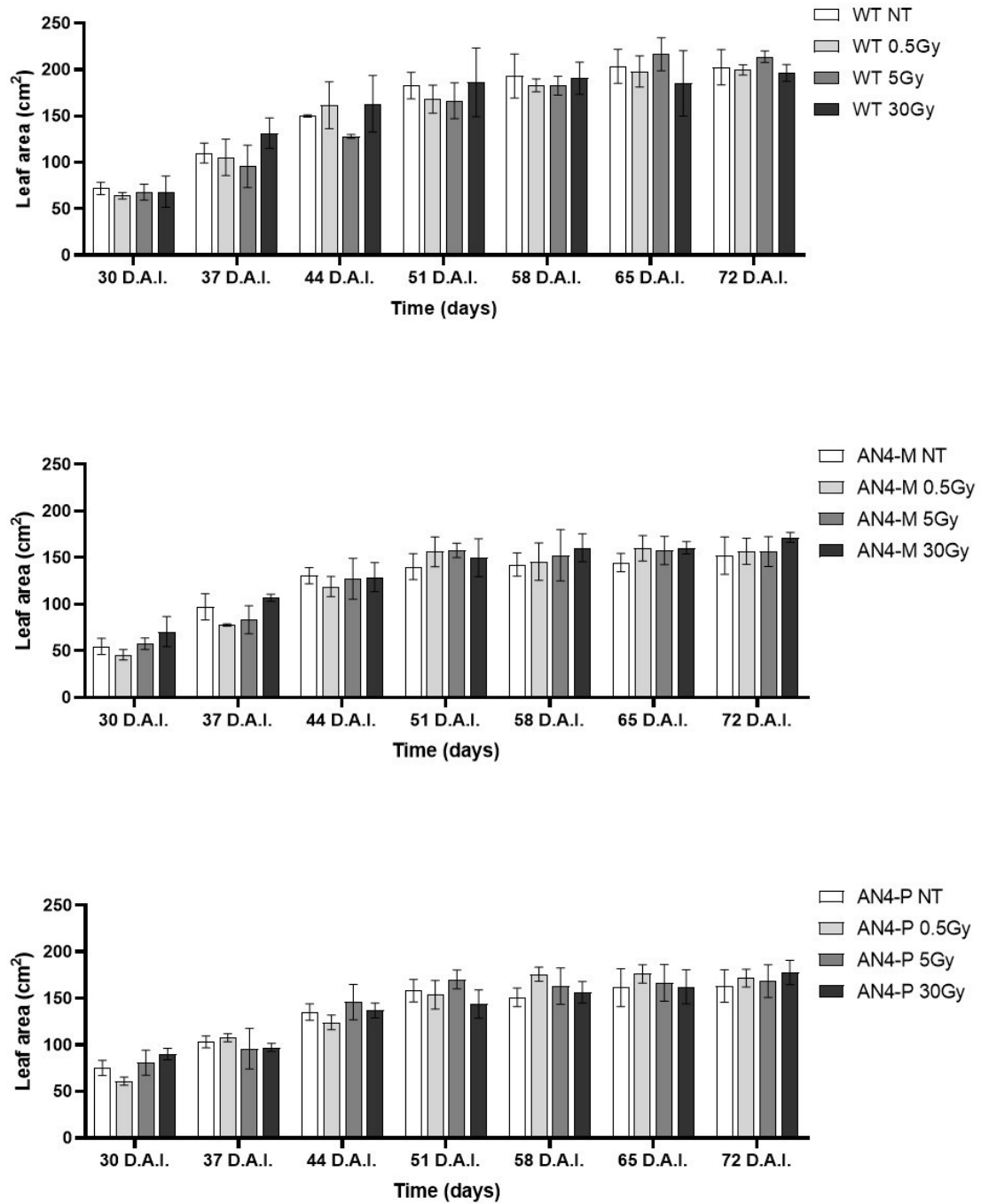


Figure 4. 32 Analysis of leaf area of wild type, AN4-M and AN4-P₂ MicroTom along the observation period upon different irradiation treatments. Statistical relevance: *: $p < 0.05$; **: $p < 0.001$; ***: $p < 0.0001$.

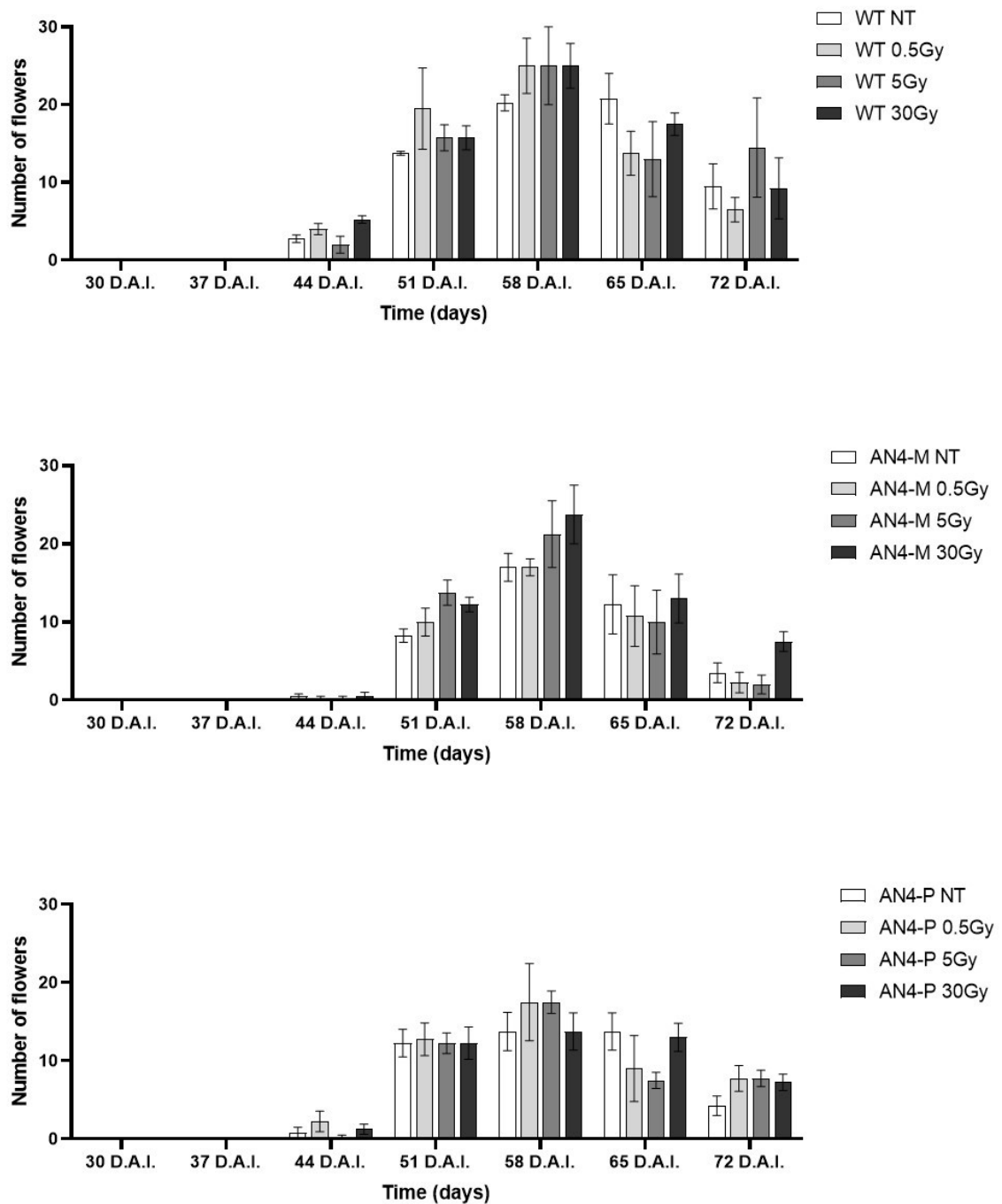


Figure 4. 33 Analysis of number of flowers of wild type, AN4-M and AN4-P₂ MicroTom along the observation period upon different irradiation treatments. Statistical relevance: *: $p < 0.05$; **: $p < 0.001$; ***: $p < 0.0001$.

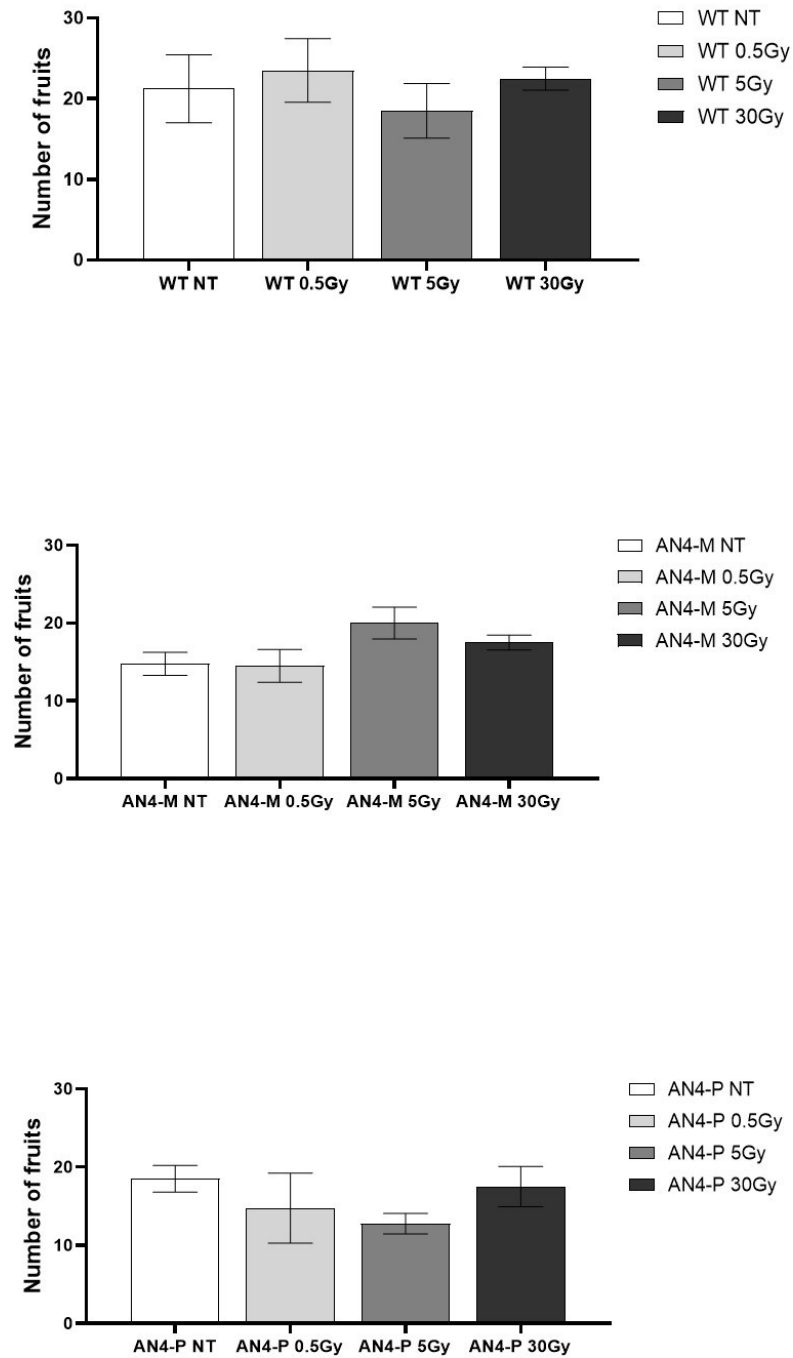


Figure 4. 34 Analysis of number of fruits of wild type, AN4-M and AN4-P₂ MicroTom plants at the end of the life cycle upon the different irradiation treatments. Statistical relevance: *: $p < 0.05$; **: $p < 0.001$; ***: $p < 0.0001$.

The values of fruits-related parameters at fruit set, were compared among the three genotypes in function of the radiation dose, as well (Figure 4.35). As already mentioned in section 4.3, the three genotypes are not significantly different for number, weight and diameter of fruits in standard conditions. Regarding these parameters, the three genotypes respond homogeneously to radiation, independently of dose supplied, as well.

On the contrary, the three genotypes are significantly different for number of seeds already in standard conditions. The offspring of gamma-irradiated seeds of the three genotypes remain different also upon radiation, with low doses (0.5 and 5 Gy) determining an increase of the number of seeds in both wild type and AN4-M plants, and a decrease in AN4-P₂. The 30 Gy dose reduced the number of seeds in both wild type and AN4-P₂ plants (percentage increase compared to non-treated is shown in the red boxes).

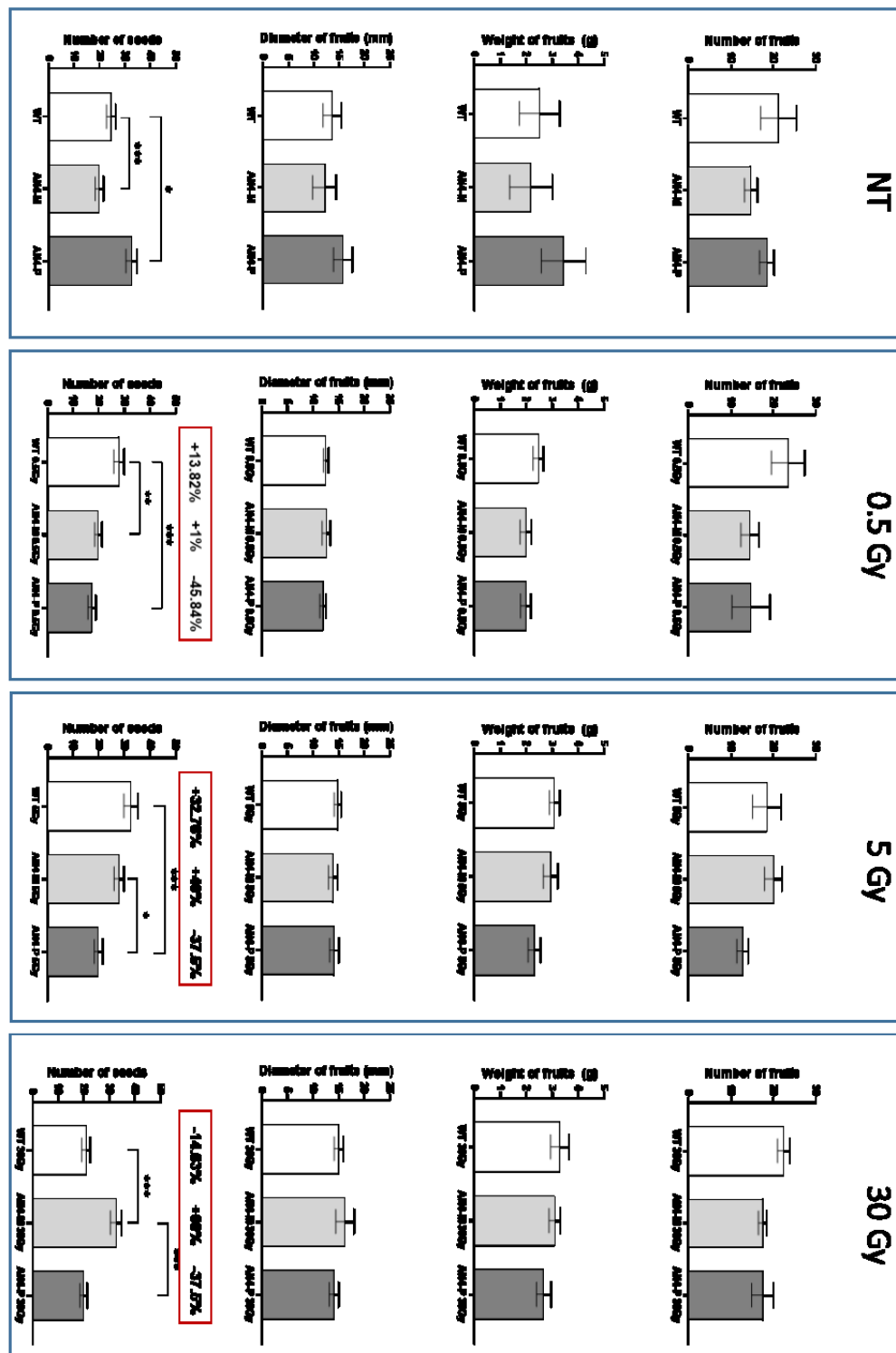


Figure 4. 35 Comparison of fruits-related parameters among the three genotypes according to the different treatments on irradiated seeds. (percentage increase compared to non-treated is shown in the red boxes).

Evaluation performed at the end of the detection period with the Multiplex Force A™ (SFR, FLAV, ANTH and NBI indices), demonstrated that the irradiation of seeds did not produce significant effects on fluorimetric parameters of offspring (Figure 4.36).

The interaction between ‘time’ and ‘gamma radiation’ variables resulted not statistically relevant and it was comprised between 3.3% and 17.4%, demonstrating that the effect of one independent variable (gamma irradiation) does not depend on the effect of the other independent variable (time). The percentage of total variation due to the ‘gamma irradiation’ variable varied between 0.28% and 6.63%, while the ‘time’ variable resulted between 10.9% and 78.3%, revealing that the variation observed in this experiment is mostly due to ‘time,’ and that ‘gamma irradiation’ of seeds only barely influenced fluorimetric parameters of offspring.

	WT NT	WT 0.5Gy	WT 5Gy	WT 30Gy	
SFR	1.65±0.03	1.36±0.12	1.51±0.1	1.71±0.1	+ ***
FLAV	-0.08±0.01	-0.11±0.01	-0.09±0.014	-0.1±0.01	+ **
ANTH	-0.11±0.003	-0.11±0.001	-0.12±0.002	-0.11±0.001	+ *
NBI	1.6±0.02	1.45±0.05	1.45±0.08	1.61±0.062	/

	AN4-M NT	AN4-M 0.5Gy	AN4-M 5Gy	AN4-M 30Gy	
SFR	1.86±0.1	1.58±0.03	1.99±0.12	1.9±0.09	- *
FLAV	-0.063±0.007	-0.001±0.01	-0.03±0.007	-0.01±0.02	- **
ANTH	-0.09±0.003	-0.08±0.008	-0.11±0.002	-0.11±0.006	- ***
NBI	1.57±0.07	1.35±0.05	1.71±0.06	1.56±0.02	

	AN4-P NT	AN4-P 0.5Gy	AN4-P 5Gy	AN4-P 30Gy	
SFR	1.97±0.08	1.7±0.09	1.74±0.15	1.81±0.13	
FLAV	-0.003±0.01	0.008±0.01	0.03±0.03	0.01±0.03	
ANTH	-0.08±0.007	-0.08±0.004	-0.07±0.01	-0.086±0.01	
NBI	1.59±0.02	1.55±0.09	1.49±0.1	1.69±0.1	

Figure 4. 36 Summary of fluorimetric results at the end of the measurement period analysed in comparison within each genotypes and according to the different treatments on irradiated seeds. Data were subjected to two-way analysis of variance (two-way ANOVA) with Tukey’s post-hoc test to determine the differences in average of all tested parameters between irradiated and non-irradiated plants. NT: non-treated.

Throughout the whole detection period, the values measured for the SFR and NBI indices (Figures 4.37 and 4.38) progressively increased from 30 to 65 DAI, as expected due to the mostly undisturbed plant growth, despite seed irradiation.

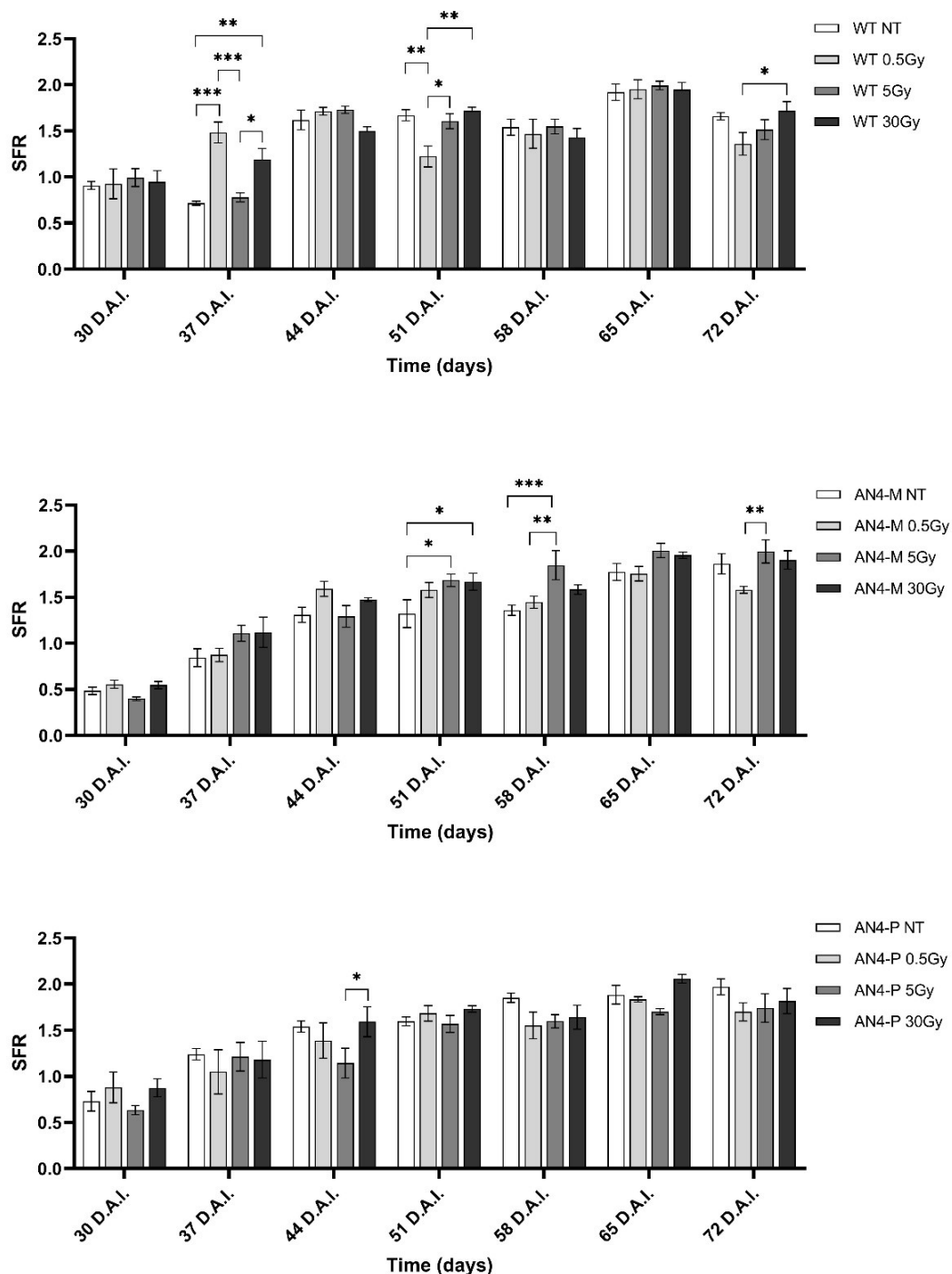


Figure 4. 37 Analysis of the SFR index of wild type, AN4-M and AN4-P₂ MicroTom along the observation period upon different irradiation treatments on irradiated seeds. Statistical relevance: *: p<0.05; **: p<0.001; ***: p<0.0001.

A sort of periodic sigmoidal-like trend of values was observed, independently of irradiation dose, for SFR mostly in wild type and AN4-M plants. If variations of SFR values were recorded in irradiated samples of the three genotypes compared to non-irradiated controls, those occurred within 58 DAI (corresponding to 58 DAS) and were, in general, positive compared to non-irradiated samples. Nevertheless, there were differences among genotypes. In wild type, an initial positive effect on SFR in 0.5 Gy and 30 Gy irradiated seeds compared to non-irradiated was noticed at 37 DAI (37 DAS) while a negative effect was observed only for the 0.5 Gy treatment, at 51 DAI (51 DAS). In 5 and 30 Gy irradiated seeds, AN4-M increases occurred at 51-58 DAI (51-58 DAS) compared to non-irradiated samples. In AN4-P₂, no statistical differences with non-treated samples were recorded throughout the whole measurement period.

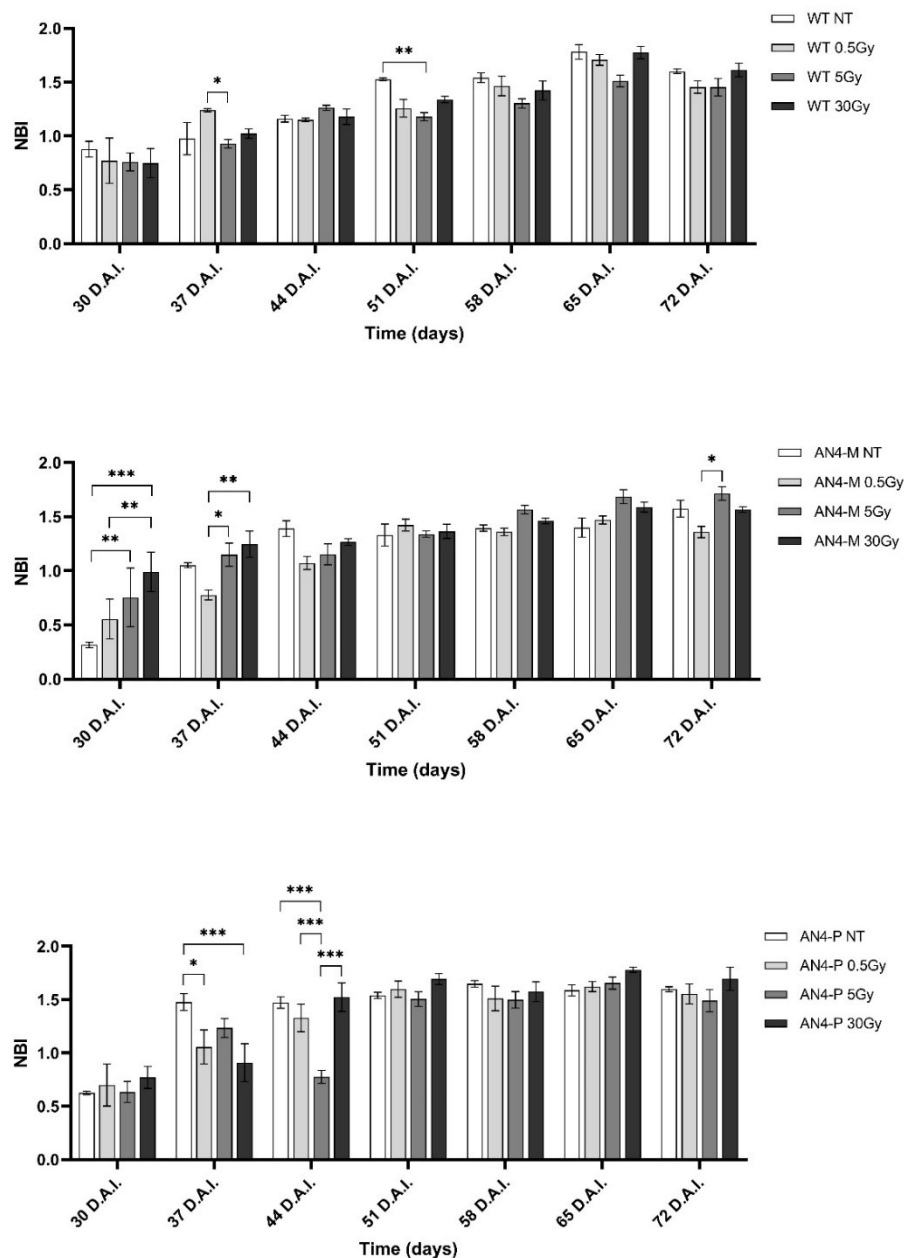


Figure 4. 38 Analysis of the NBI index of wild type, AN4-M and AN4-P₂ MicroTom along the observation period upon different irradiation treatments on irradiated seeds. Statistical relevance: *: $p < 0.05$; **: $p < 0.001$; ***: $p < 0.0001$.

Variations of NBI values recorded in irradiated vs non-irradiated samples occurred irrespective of genotypes. Nevertheless, there were differences among genotypes. In wild type, a negative effect was observed for 5 Gy samples at 51 DAI (51 DAS). In 5 and 30 Gy irradiated AN4-M samples, increases compared to non-treated samples occurred at 30 DAI (30 DAS). In AN4-P₂, a significant decrease was found at 37 DAI (37 DAS) for 0.5 and 30 Gy irradiated samples while at 44 DAI (44 DAS) the 5 Gy dose caused a significant decrease of the index.

Fluorescent emission measurements (Fv/Fm and Y_Fm) were performed in order to track the performances of the photosynthetic apparatus and its capacity to recovery from the stress that plants have been subjected to. The photosynthetic maximum photochemical efficiency (Fv/Fm) and the maximum fluorescent yield (Y_Fm) resulted in the range (0.7/0.9) described for healthy leaves by the Plant Stress Kit (PSKII) manufacturer for any dose within each genotype along time, indicating no stress induction by irradiation. Nevertheless, some statistically relevant differences were noted for each genotype especially at the end of life cycle (Figure 4.39 and Figure 4.40). In particular, both wild type and AN4-M plants experienced a significant decrease of the Fv/Fm index at 65 DAI (65 DAS) when subjected to the 5Gy dose. At 72 DAI (72 DAS) the AN4-M genotype subjected to the 30 Gy showed a statistically significant decrease while the AN4-P₂ genotype had an increase when subjected to the 5 Gy dose. In relation to the Y_Fm index, the 5 Gy dose caused a decrease of the index starting from 58 DAI (58 DAS) to 72 DAI (72DAS). In addition, during the same period the AN4-M showed a decrease for plants subjected to 30 Gy at 58 DAI (58 DAS), 5 and 30 Gy at 65 DAI (65 DAS) and 5 Gy at 72 DAI (72 DAS). Conversely, the 0.5 Gy dose negatively affected AN4- P₂ plants only at 65 DAI (65 DAS).

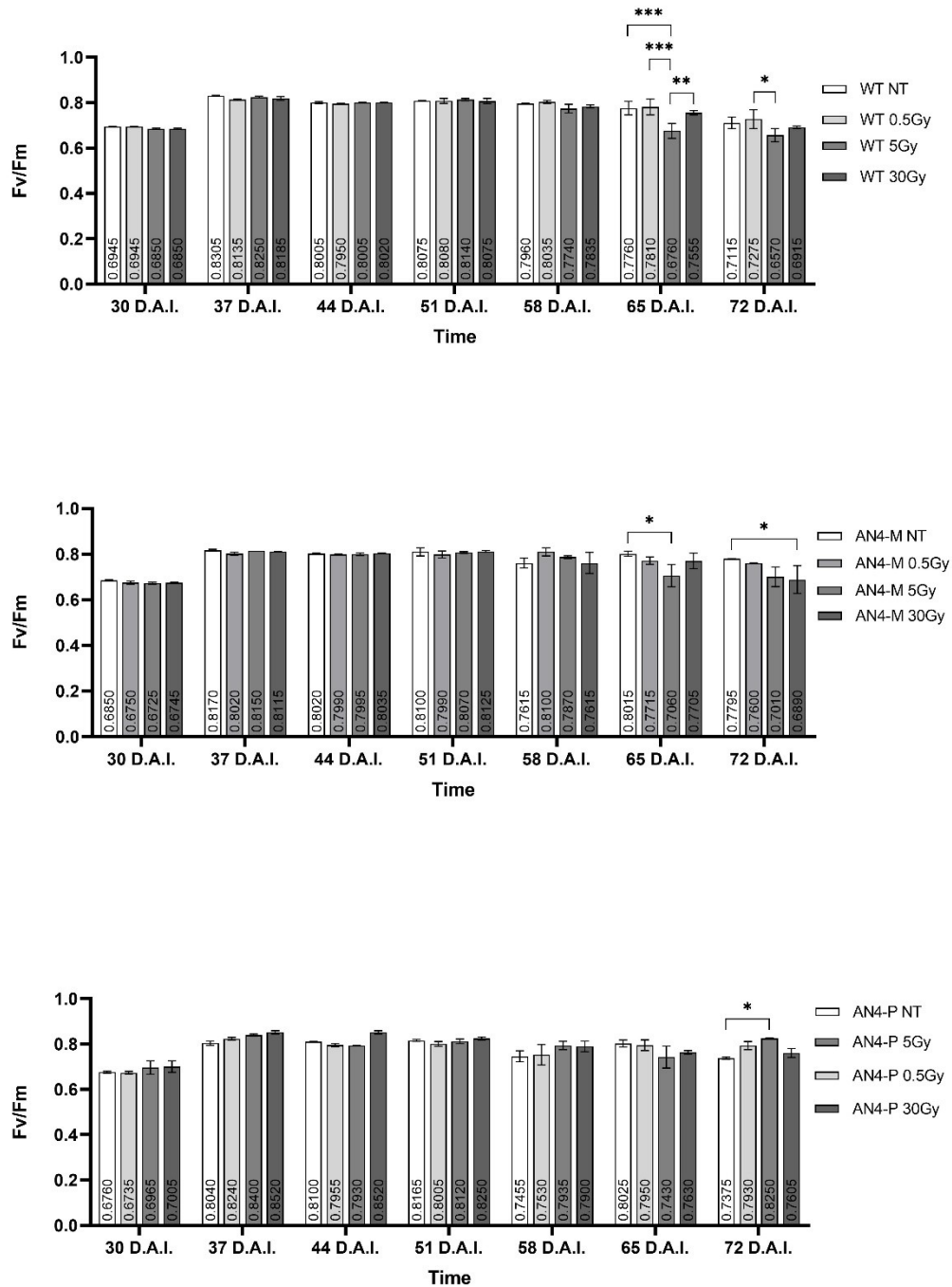


Figure 4. 39 Analysis of the Fv/Fm index of wild type, AN4-M and AN4-P₂ MicroTom along the observation period upon different irradiation treatments on irradiated seeds. Statistical relevance: *: $p < 0.05$; **: $p < 0.001$; ***: $p < 0.0001$.

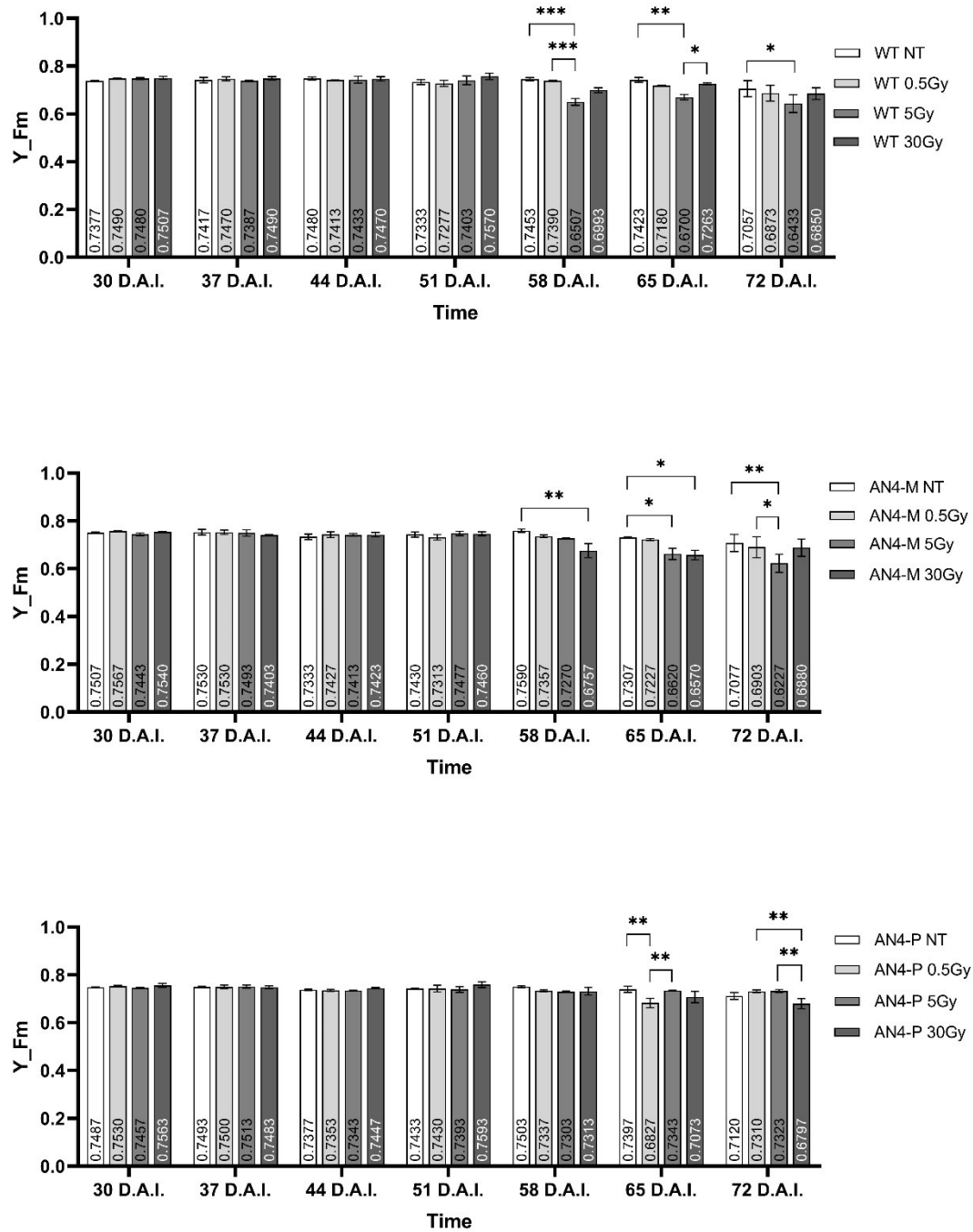


Figure 4. 40 Analysis of the Y_Fm index of wild type, AN4-M and AN4-P₂ MicroTom along the observation period upon different irradiation treatments on irradiated seeds. Statistical relevance: *: p<0.05; **: p<0.001; ***: p<0.0001.

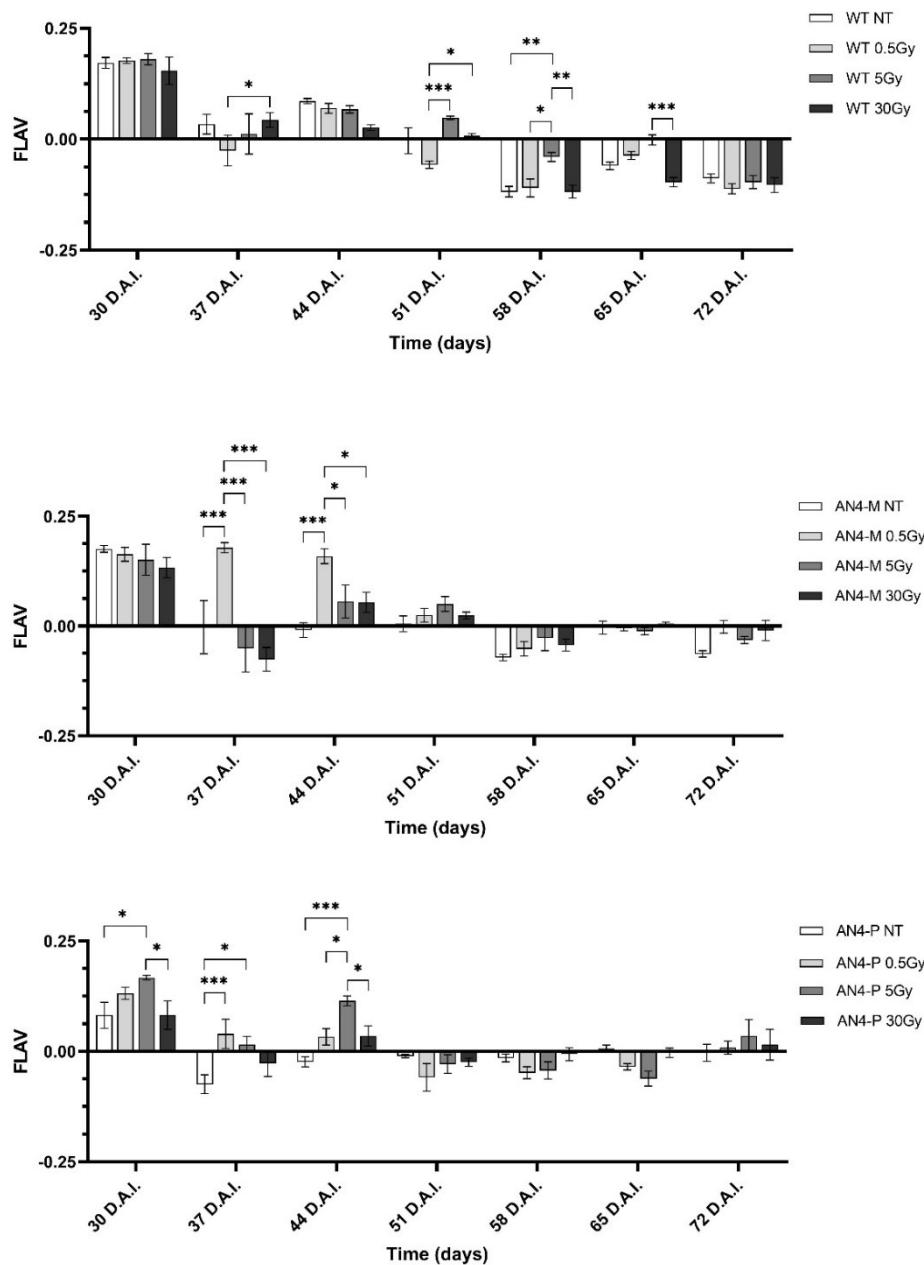


Figure 4. 41 Analysis of the FLAV index of wild type, AN4-M and AN4-P₂ MicroTom along the observation period upon different irradiation treatments on irradiated seeds. Statistical relevance: *: $p < 0.05$; **: $p < 0.001$; ***: $p < 0.0001$.

The FLAV index values recorded throughout the measurement period showed a periodic sigmoidal-like shape trend for all the genotypes with several inflection points.

The inversion of data towards negative values occurred at 44-51 DAI (44-51 DAS) in wild type and P₂ plants, and 51-58 DAI (51-58 DAS) in M plants (Figure 4.41). This inversion is compatible with the shift from the vegetative state to the flowering time, with metabolic changes that possibly reduce the metabolic flux of secondary metabolites. After this ‘inversion point’,

FLAV values are generally higher for transgenic plants compared to wild type. In particular, the irradiation procedure resulted in a positive effect of the 5 Gy dose at 58 DAI in wild type plants. A similar effect was found between 37 and 44 DAI for AN4-M plants subjected to the 0.5 Gy dose. Moreover, the 5 Gy irradiation resulted in an increase of the FLAV index also in AN4-P₂ plants between 30 and 44 DAI.

As somehow expected due to the virtual absence of anthocyanins bioaccumulation in wild type MicroTom as a consequence of natural gene mutations that block biosynthesis, the ANTH index values defined differential curves for the wild type compared to the two transgenic genotypes (Figure 4.42).

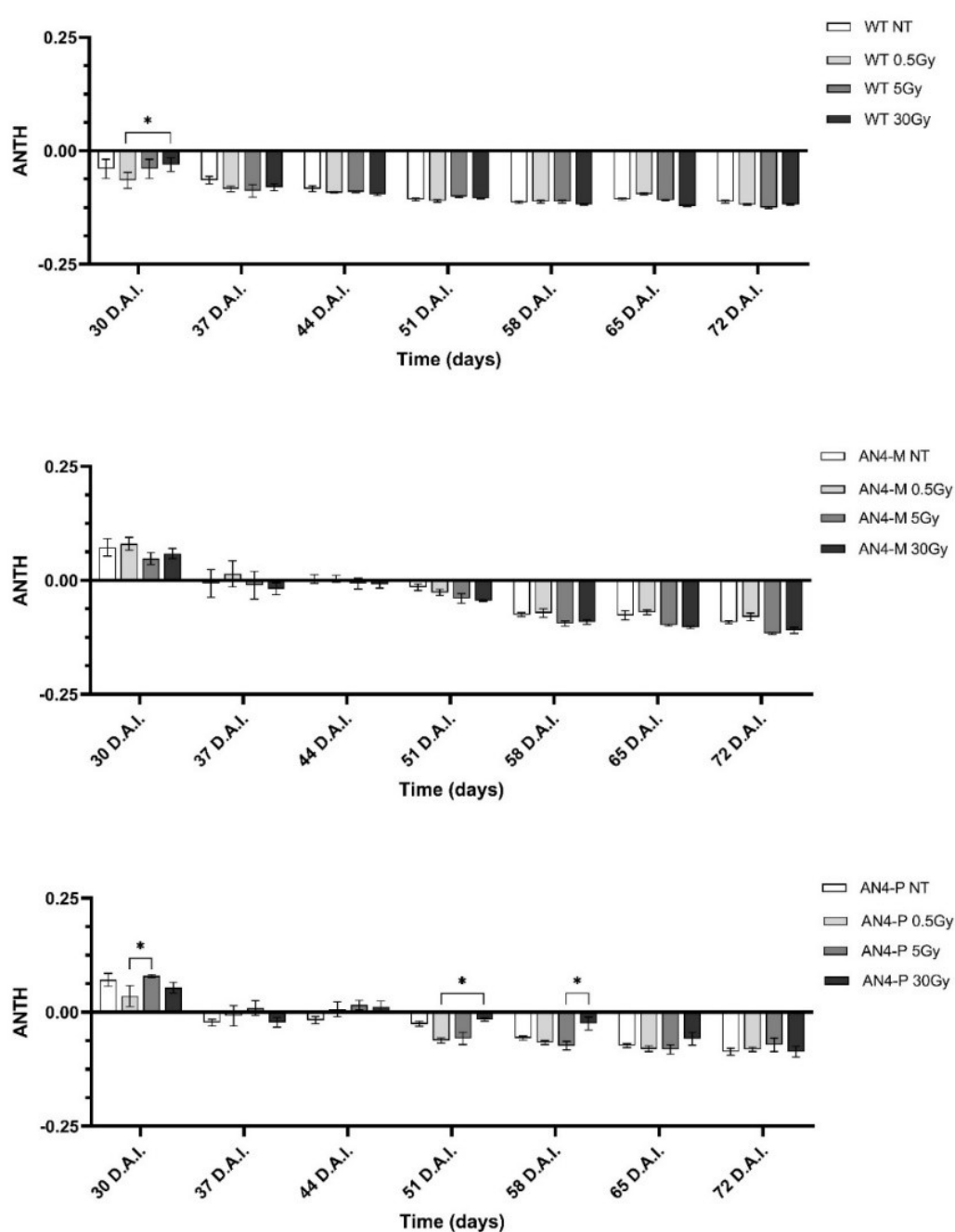


Figure 4. 42 Analysis of the ANTH index of wild type, AN4-M and AN4-P₂ MicroTom along the observation period upon different irradiation treatments on irradiated seeds. Statistical relevance: *: $p < 0.05$; **: $p < 0.001$; ***: $p < 0.0001$.

Indeed, while ANTH values were constantly negative throughout life cycle of wild type offspring with no statistical relevance between irradiated and non-irradiated seeds, indicating absence of anthocyanins, AN4-M and AN4-P₂ offspring of both irradiated and non-irradiated seeds were characterized by a sigmoidal curve with a main inflection point (corresponding to inversion to negative values) around 37-51 DAI (37-51 DAS). Also in this case, the main inversion point corresponded to the shift from the vegetative state to the flowering time, with metabolic changes possibly redirecting secondary metabolic pathways.

4.6.2 Biometric and fluorimetric characterization of plants irradiated at 30 DAS

The 30 days after sowing (DAS) life stage represents the crucial period when MicroTom has completed approximately one third of its life cycle and the first true leaves are fully developed. The PCA analysis was performed on the dataset obtained from plants irradiated with 0.5 and 5 Gy doses (Figure 4.43), due to the presence of missing values (i.e., number of fruits) in the 30 Gy data matrix. The contributions of the first two PCs were 32.04% (PC1) and 16.33% (PC2), respectively, and their sum explained the 48.73% of the phenotypic variation. As showed in the Loadings plot (Figure 4.43A), NBI, FLAV, ANTH, and number of flowers resulted positively correlated with the PC1, while morphometric parameters such as leaf area, diameter of fruits, plant height, number of fruits, number of leaves, number of seeds, weight of fruits were negatively correlated with it. PC2 was positively dominated by SFR, NBI, FLAV, leaf area, diameter of fruits, and plant height, while ANTH, number of flowers, number of seeds, and weight of fruits negatively dominate PC2. Therefore, the weight of fruits and the number of seeds resulted negatively correlated with both PC1 and PC2, while SFR, NBI and FLAV indices were positively correlated with both PC1 and PC2.

As in the case of the irradiation performed on seeds, the score plot and the biplot (Figure 4.43B and Figure 4.43C) showed a specific localization of outcomes in function of the three different genotypes. Indeed, the scores of wild type plants clustered in the left quadrants of the plot (negative variations of PC1), while scores related to AN4-M clustered near the origin of the axes and along the y axes, demonstrating little contribution of AN4-M plants to the overall PC1 variation but both positive and negative correlation to PC2. The scores of the AN4-P₂ plants clustered in the right quadrants of the plot (positive variations of the PC1). This scenario shows

that, as expected due to the *PhAN4* transgenesis, wild type and transgenic genotypes can be discriminated on the basis of the indices positively correlated to FLAV and ANTH.

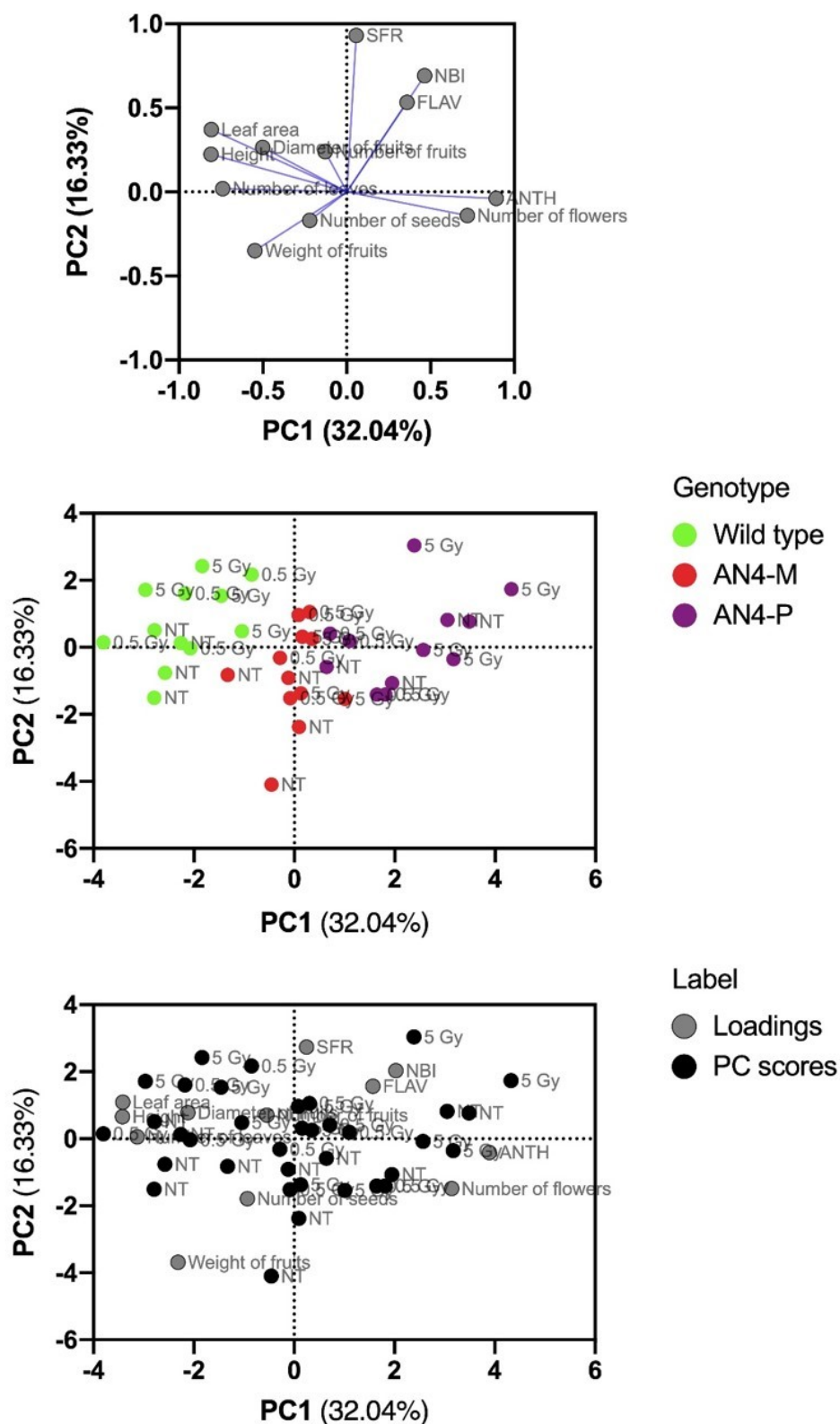


Figure 4. 43 Principal component analysis on morphometric and fluorimetric parameters obtained from plants irradiated at 30 DAS. Results are graphically reported using the Loadings plot with all the parameters considered (A), the PC scores plot with wild type and AN4 plants (B) and the biplot that contains the information presented in both the Loadings plot and the PC scores plot at the same time (C).

Non-treated wild type plants showed negative correlation with both PC1 and PC2, while irradiated wild type plants, irrespective of doses, showed negative correlation with PC1 and positive with PC2 (clustering in the upper left quadrant). Finally, AN4-P₂ plants showed positive correlation with PC1 and positive or negative correlation with PC2 (clustering both in the upper-right and lower-right corner).

The analysis of variance (two-way Anova) performed at the end of the detection period revealed that the interaction between ‘time’ and ‘treatment’ variables was not statistically significant and that it did not exceed the 16.3% of the total variation. The % of variation ascribed to the ‘time’ variable was included between 61.4% and 91.7%, confirming that the most of the variation observed in the experiment is mainly due to the variable ‘time’ than to the variable ‘gamma irradiation’ (as in the case of irradiation of seeds) and that the latter only partially influenced plants in their morphometry. The % of total variation ascribed to the irradiation procedure resulted included between 1.21% and 22%, higher than that observed for offspring of irradiated seeds (from 0.002 to 2.31% as described in section 4.6.1), demonstrating that gamma rays supplied at different developmental stages (seed *vs* plant at 30 DAS) can affect plants in a different manner. Indeed, considering the values of the parameters at the end of the detection period (red ripe fruit stage), irradiation of plants at 30 DAS produced statistically relevant effects on a higher number of biometric parameters compared to irradiated seeds, as summarized in Figure 4.44. In addition, and differently from irradiated seeds, the irradiation of plants at 30 DAS with 30 Gy determined an overall significant negative effects on the whole of both vegetative and yield traits, independently from the genotype. Due to the constant of this result for every variable, for the sake of simplicity, the data related to the effects of the 30 Gy dose will be generally omitted afterwards.

Irradiated wild type plants showed significant positive variation in number of leaves, leaf area and number of seeds (0.5 and 5 Gy doses; Figure 4.44). Irradiated AN4-M plants showed no variation of parameters compared to non-irradiated controls (Figure 4.44).

	WT NT	WT 0.5Gy	WT 5Gy	WT 30Gy	
Height (cm)	11.56±0.17	10.89±0.26	11.7±0.59	9.69±0.18	+ ***
Number of leaves	27.6±0.92	33±1.3	33.6±1.6	28.6±1.16	+ **
Leaf area (mm ²)	194.65±12.67	229.45±14.1	219.33±14.98	57.05±4.55	+ *
Number of flowers	15.4±0.5	16.6±0.24	17.2±0.66	2±0.63	/
Number of fruits	16.4±1.02	16.2±0.37	15.4±0.67	/	- *
Weight of fruits (g)	2.84±0.25	2.54±0.22	2.85±0.23	/	- **
Diameter of fruits (mm)	17.48±0.68	19.88±1.33	18.6±0.54	/	- ***
Number of seeds	17.7±1.03	21.2±0.75	19.7±0.47	/	

	AN4-M NT	AN4-M 0.5Gy	AN4-M 5Gy	AN4-M 30Gy
Height (cm)	8.5±0.52	9.58±0.13	9.3±0.28	6.54±0.28
Number of leaves	26±2.12	28±2.75	27±3.47	13±1.22
Leaf area (mm ²)	116.98±6.79	114.5±6.11	124.45±7.51	83.76±3.49
Number of flowers	18±0.83	17.6±0.81	18.4±0.6	3±0.44
Number of fruits	16.4±0.4	16.4±1.2	16±0.8	/
Weight of fruits (g)	2.78±0.19	2.51±0.17	2.3±0.15	/
Diameter of fruits (mm)	16.15±0.57	15.89±0.54	16.24±0.77	/
Number of seeds	21.5±0.63	22.2±0.48	21.7±0.3	/

	AN4-P NT	AN4-P 0.5Gy	AN4-P 5Gy	AN4-P 30Gy
Height (cm)	9.02±0.32	9.74±0.54	7.66±0.25	7.58±0.19
Number of leaves	18.2±1.8	20.6±1.2	14.8±1.15	10.4±0.5
Leaf area (mm ²)	114.02±2.3	125.49±0.87	104.55±5.79	68.49±1.42
Number of flowers	19.2±0.37	19.2±0.37	18.8±0.73	2.6±0.5
Number of fruits	15.2±0.37	16.4±0.5	16±0.54	/
Weight of fruits (g)	2.05±0.26	1.61±0.2	1.25±0.24	/
Diameter of fruits (mm)	15.29±1.06	14.63±0.89	13.29±1.46	/
Number of seeds	17.6±0.26	20±0.39	20.9±0.52	/

Figure 4. 44 Summary of morphometric results at the end of the measurements period analysed in comparison within each genotypes and according to the different treatments supplied to plants at 30 DAS. Data were subjected to two-way analysis of variance (two-way ANOVA) with Tukey's post-hoc test to determine the differences in average of all tested parameters between irradiated and non-irradiated plants. NT: not-treated.

Analysing parameters throughout the detection period, with the mentioned exception of the plants irradiated with 30 Gy dose, no significant variations were found in plant height for irradiated wild type plants compared to non-treated plants, independently of the absorbed dose (Figure 4.45). Positive significant effects on plant height were seen only at 28 DAI (58 DAS) on AN4-M plants irradiated with the 0.5 Gy dose. AN4-P₂ plants showed variable both negative and positive effects on plant height after irradiation. In particular, after a negative variation registered at 14 DAI (44 DAS) for AN4-P₂ plants subjected to the 5 Gy dose, and an overall positive effect

seen from 21 to 35 DAI (51- 65 DAS) after the 0.5 and/or 5 Gy doses, a negative variation of height was recorded again in AN4-P₂ plants that had received the 5 Gy dose (Figure 4.45).

Variation of number of leaves initiated in both wild type and AN4-P₂ plants on the 35 DAI (65 DAS), with positive effects at 0.5 and 5 Gy for wild type registered also at 42 DAI (end of the detection period) and variable effects for AN4-P₂, ending with a negative variation 42 DAI (end of the detection period) (Figure 4.46).

With the exception of the 30 Gy dose that determined always negative variations, significant positive variations of leaf area were visible in wild type plants from 28 DAI to 35 DAI (58-65 DAS) for the 0.5 Gy dose compared to non-treated samples. No significant variation of leaf area was recorded for irradiated AN4-M plants compared to non-treated controls throughout the whole measurement period. Slight variations of leaf area were recorded for the AN4-P₂ throughout the measurements (Figure 4.47). With the only exception of plants that had received the 30 Gy dose, none of the plants, independently of genotypes, showed statistical variation in the number of flowers, number and diameter of fruits compared to the corresponding non-treated controls throughout the measurements (Figure 4.44 and from Figure 4.48 to Figure 4.52).

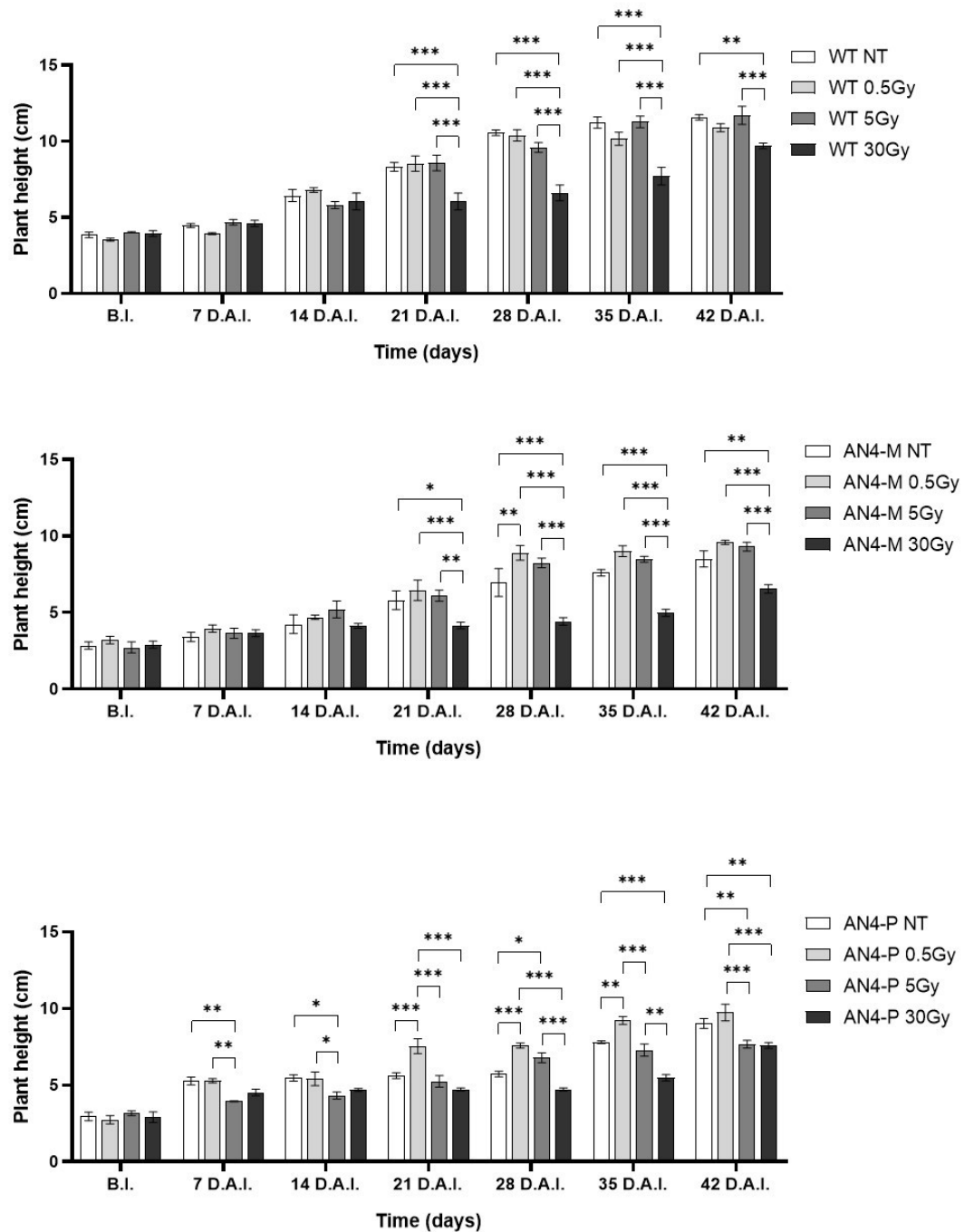


Figure 4. 45 Analysis of plant height of wild type, AN4-M and AN4-P₂ MicroTom along the observation period upon different irradiation treatments supplied to plants at 30 DAS. Statistical relevance: *: $p < 0.05$; **: $p < 0.001$; ***: $p < 0.0001$.

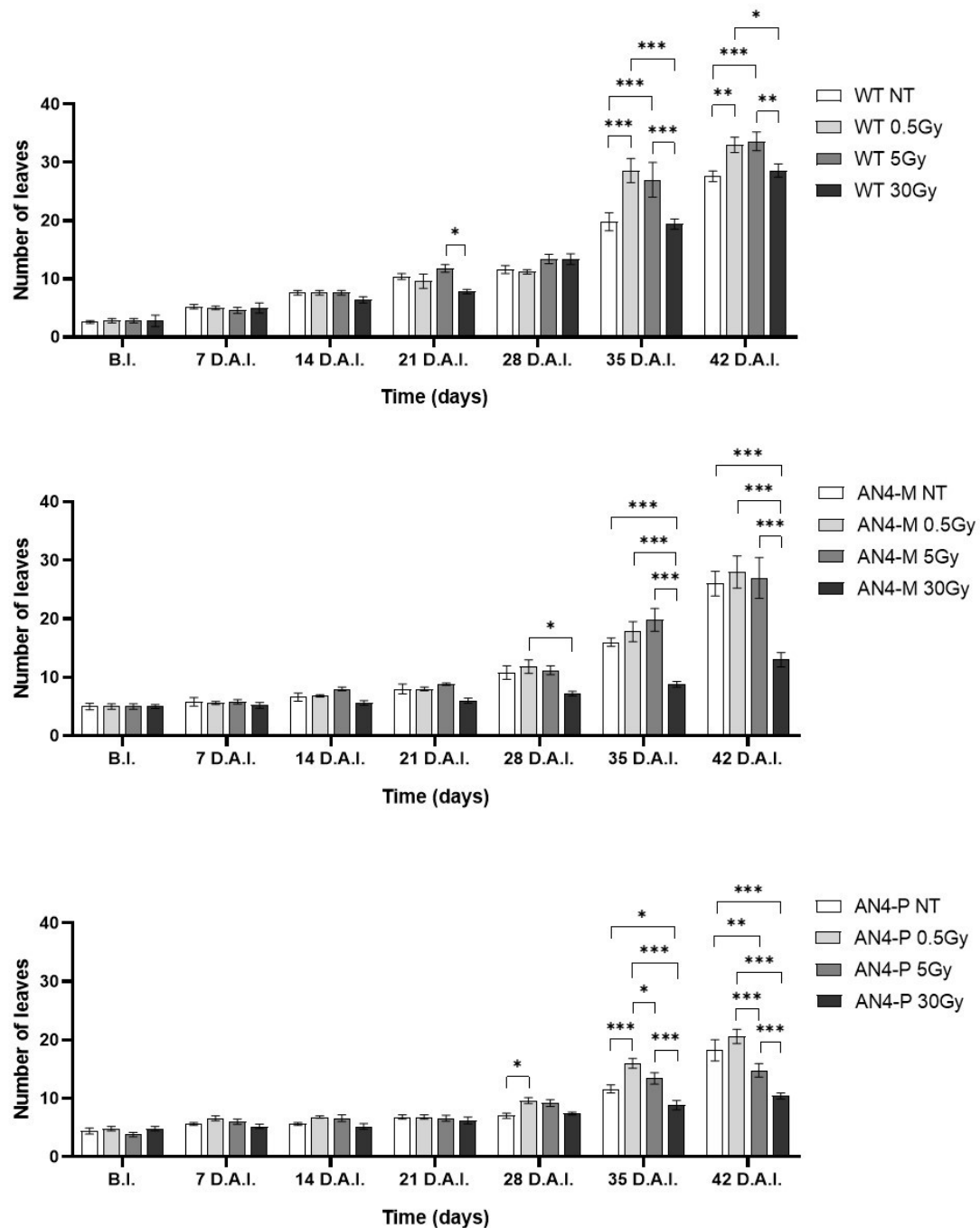


Figure 4. 46 Analysis of number of leaves of wild type, AN4-M and AN4-P₂ MicroTom along the observation period upon different irradiation treatments supplied to plants at 30 DAS. Statistical relevance: *: $p < 0.05$; **: $p < 0.001$; ***: $p < 0.0001$.

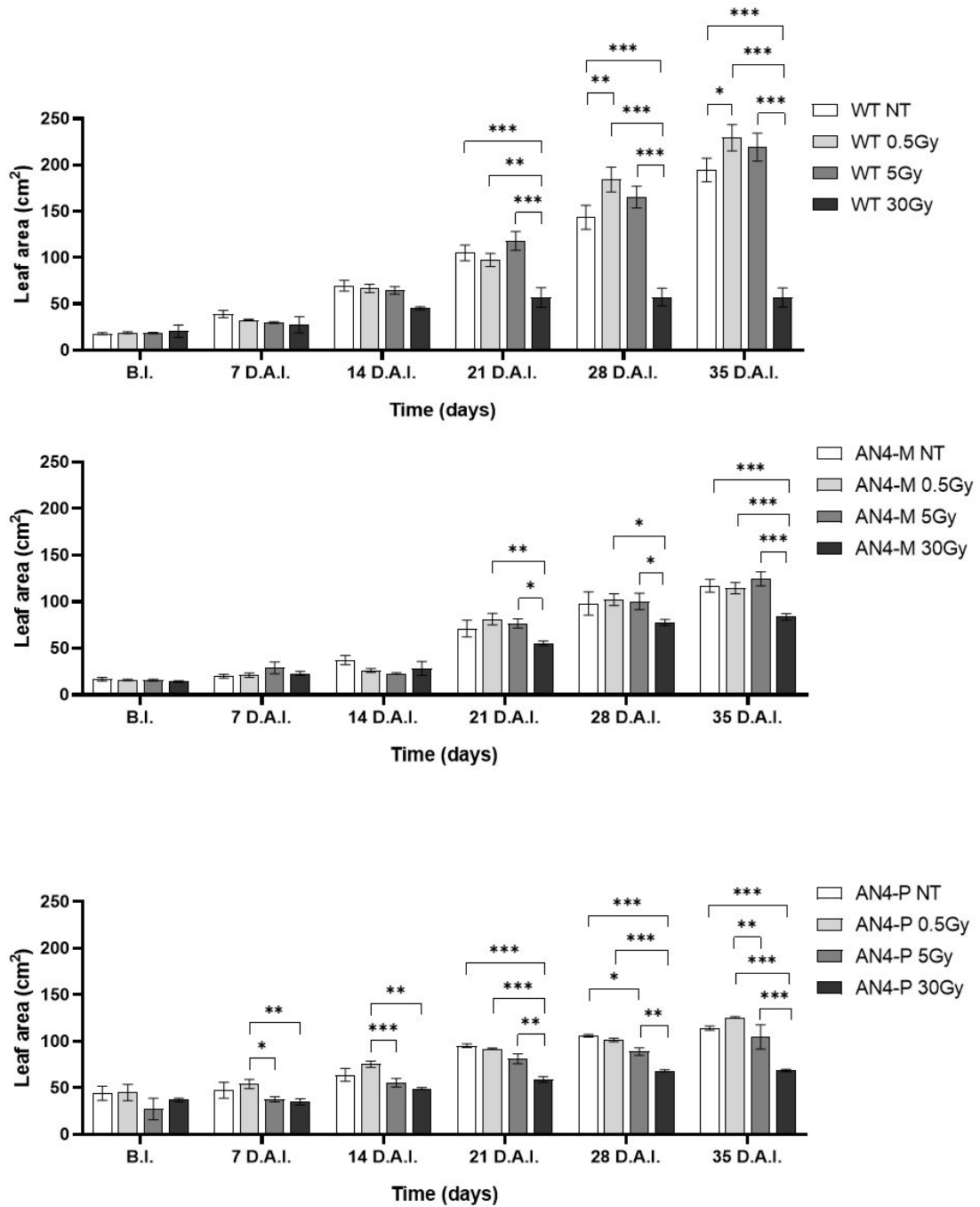


Figure 4. 47 Analysis of leaf area of wild type, AN4-M and AN4-P₂ MicroTom along the observation period upon different irradiation treatments supplied to plants at 30 DAS. Statistical relevance: *: $p < 0.05$; **: $p < 0.001$; ***: $p < 0.0001$.

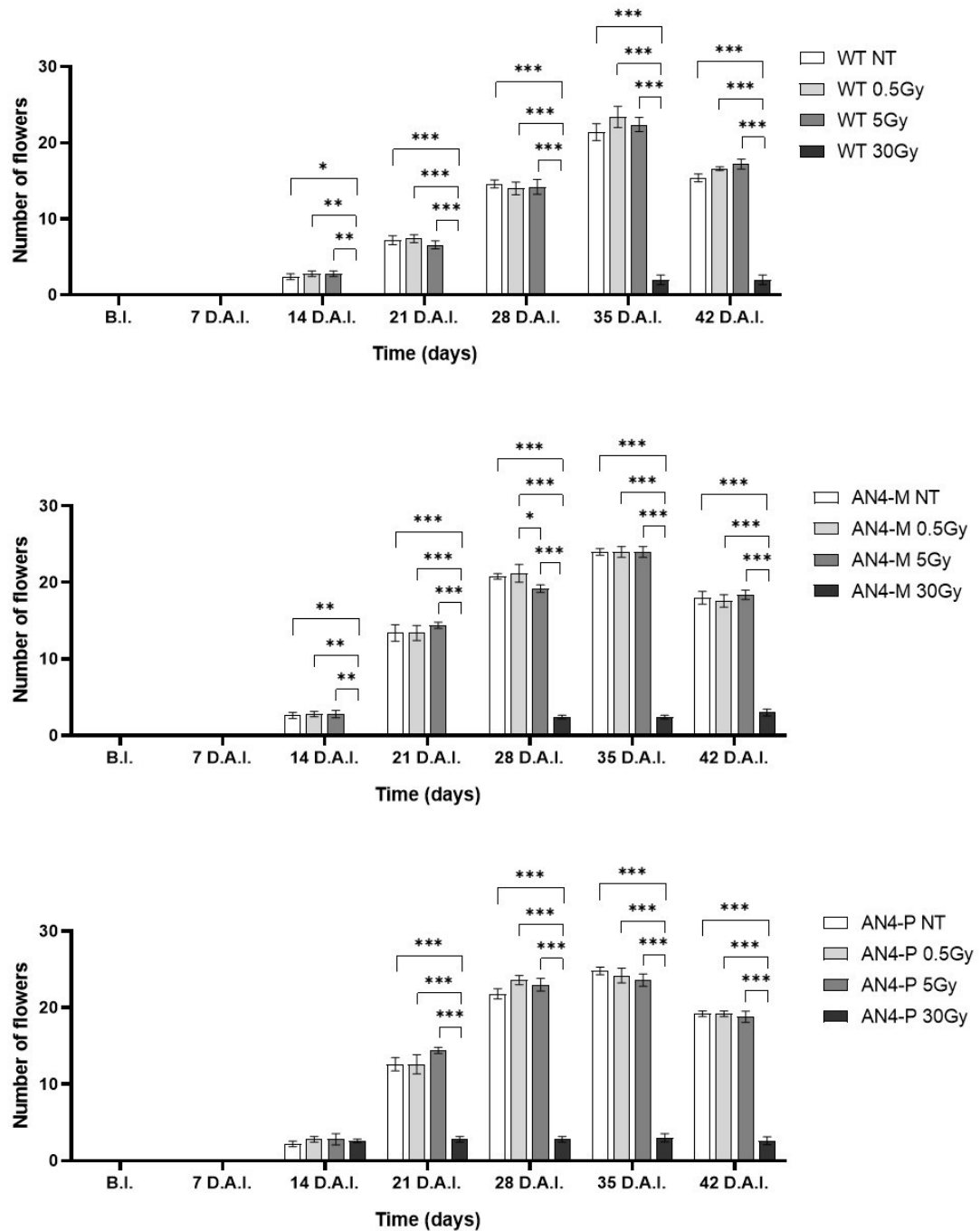


Figure 4. 48 Analysis of number of flowers of wild type, AN4-M and AN4-P₂ MicroTom along the observation period upon different irradiation treatments supplied to plants at 30 DAS. Statistical relevance: *: $p < 0.05$; **: $p < 0.001$; ***: $p < 0.0001$.

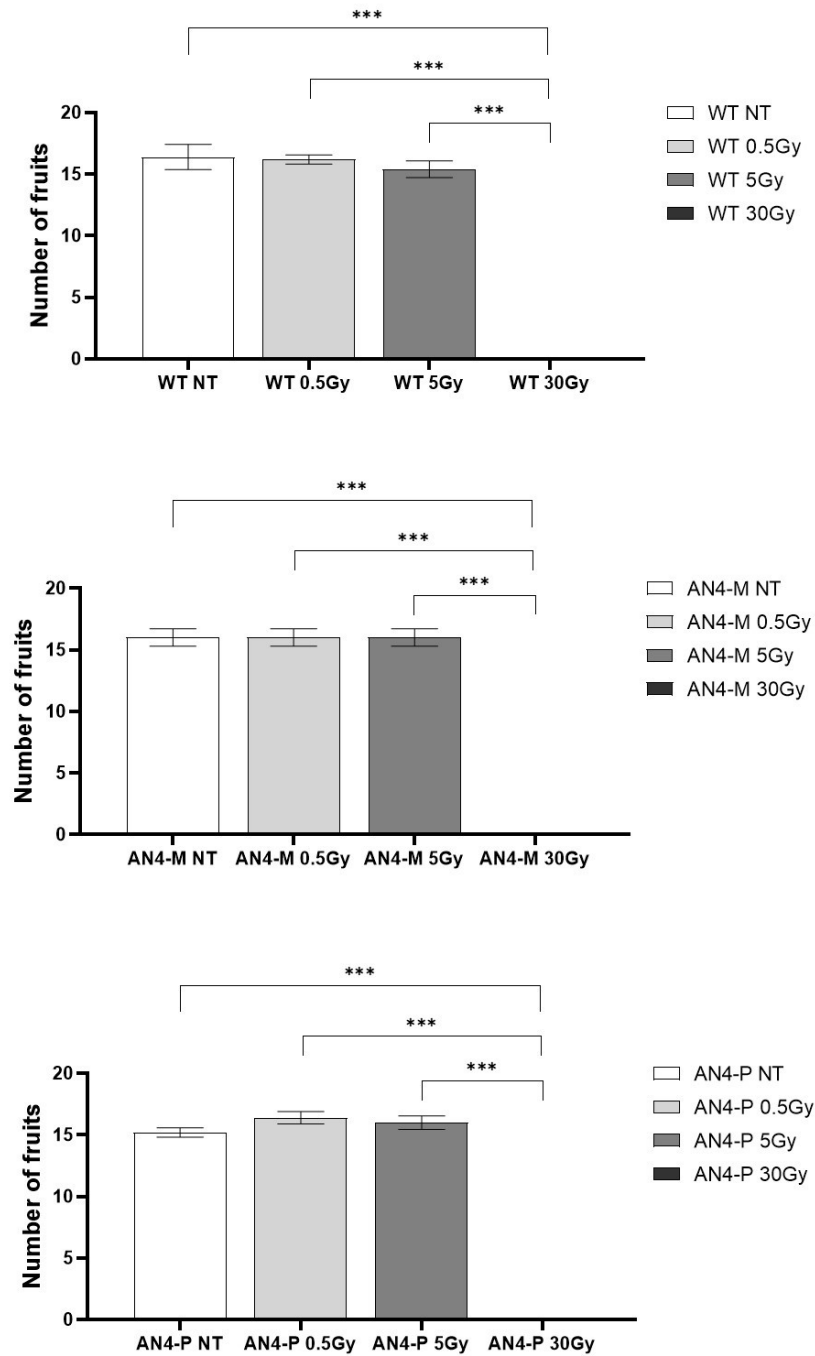


Figure 4. 49 Analysis of number of fruits of wild type, AN4-M and AN4-P₂ MicroTom at the end of the life cycle upon different irradiation treatments supplied to plants at 30 DAS. Statistical relevance: *: $p < 0.05$; **: $p < 0.001$; ***: $p < 0.0001$.

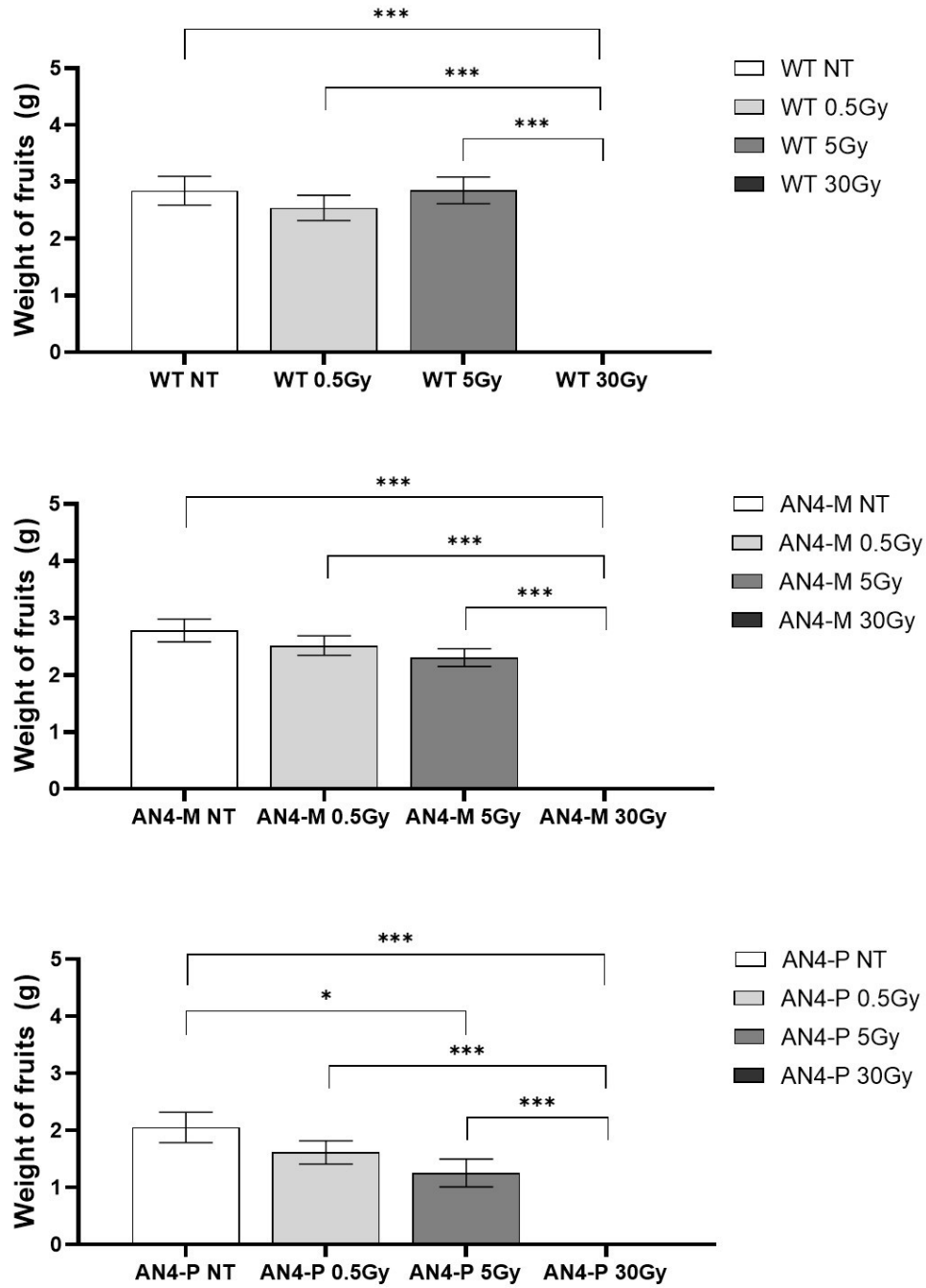


Figure 4. 50 Analysis of weight of fruits of wild type, AN4-M and AN4-P₂ MicroTom at the end of the life cycle upon different irradiation treatments supplied to plants at 30 DAS. Statistical relevance: *: $p < 0.05$; **: $p < 0.001$; ***: $p < 0.0001$.

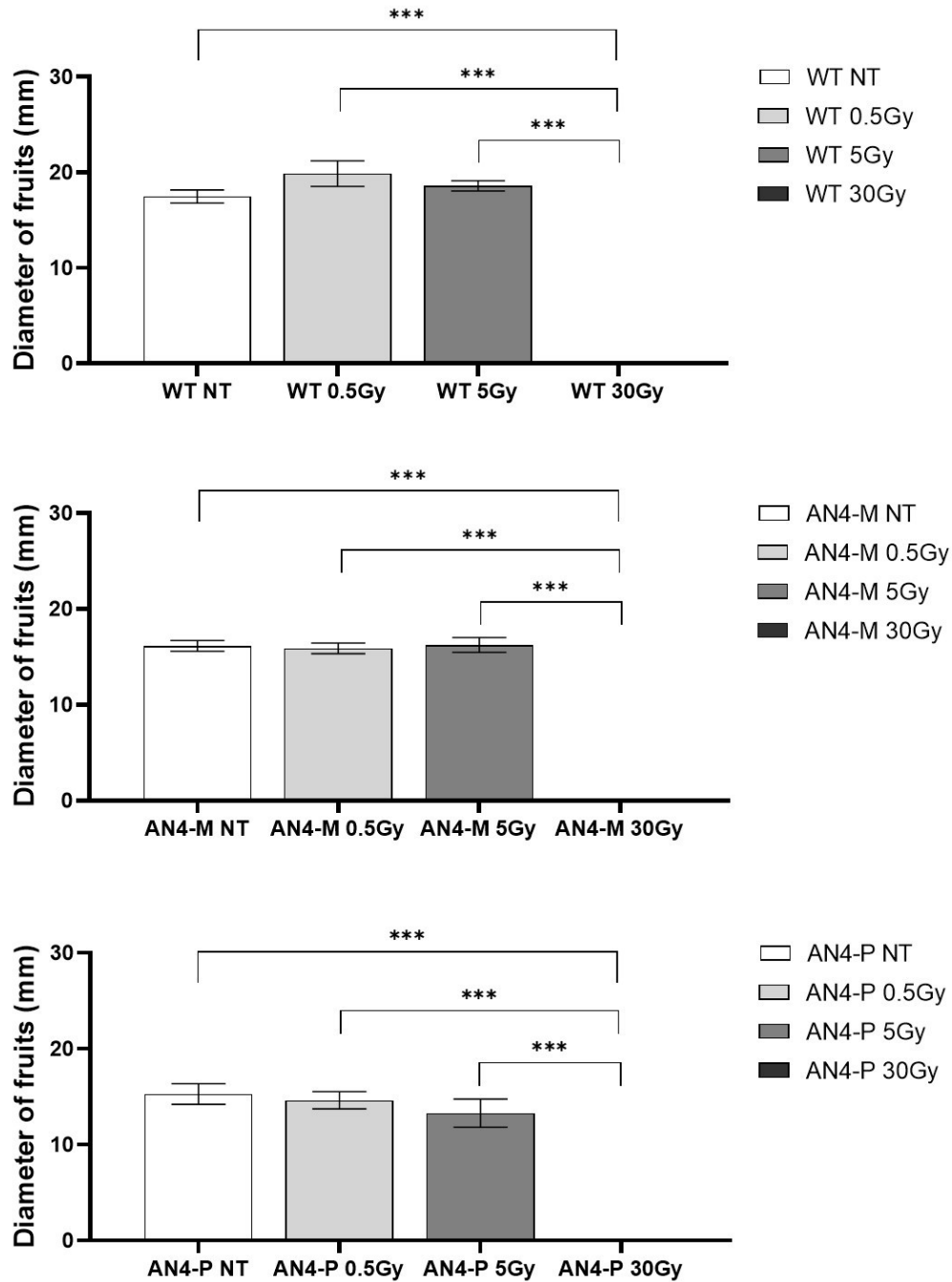


Figure 4. 51 Analysis of diameter of fruits of wild type, AN4-M and AN4-P₂ MicroTom at the end of the life cycle upon different irradiation treatments supplied to plants at 30 DAS. Statistical relevance: *: $p < 0.05$; **: $p < 0.001$; ***: $p < 0.0001$.

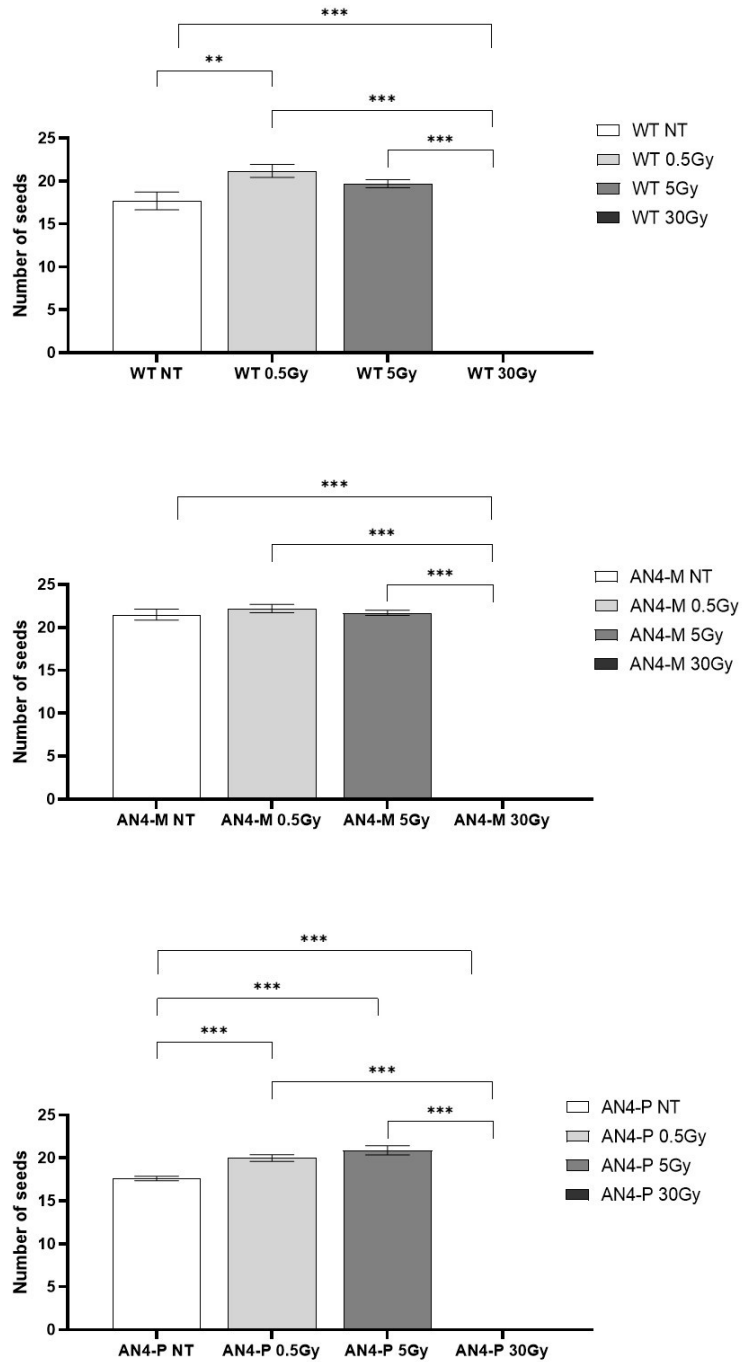


Figure 4. 52 Analysis of number of seeds of wild type, AN4-M and AN4-P₂ MicroTom at the end of the life cycle upon different irradiation treatments supplied to plants at 30 DAS. Statistical relevance: *: p<0.05; **: p<0.001; ***: p<0.0001.

The values of fruits-related parameters at fruit set, were compared among the three genotypes in function of the radiation dose, as well (Figure 4.53). As already mentioned in section 4.3 and as seen in section 4.6.1, the three genotypes are not significantly different for number, weight and diameter of fruits in standard conditions. Differently from the results obtained from irradiated seeds, 0.5 and 5 Gy doses negatively affected the weight of fruits of AN4-P₂ plants that resulted significantly lighter than wild type and AN4-M fruits. The diameter of fruits was negatively influenced in AN4-M plants after 0.5 Gy and in AN4-P₂ plants after 0.5 and 5 Gy doses. Gamma rays produced an increase of the number of seeds in all the three genotypes compared to non-treated controls both after 0.5 and 5 Gy doses (percentage increase compared to non-treated is shown in the red boxes).

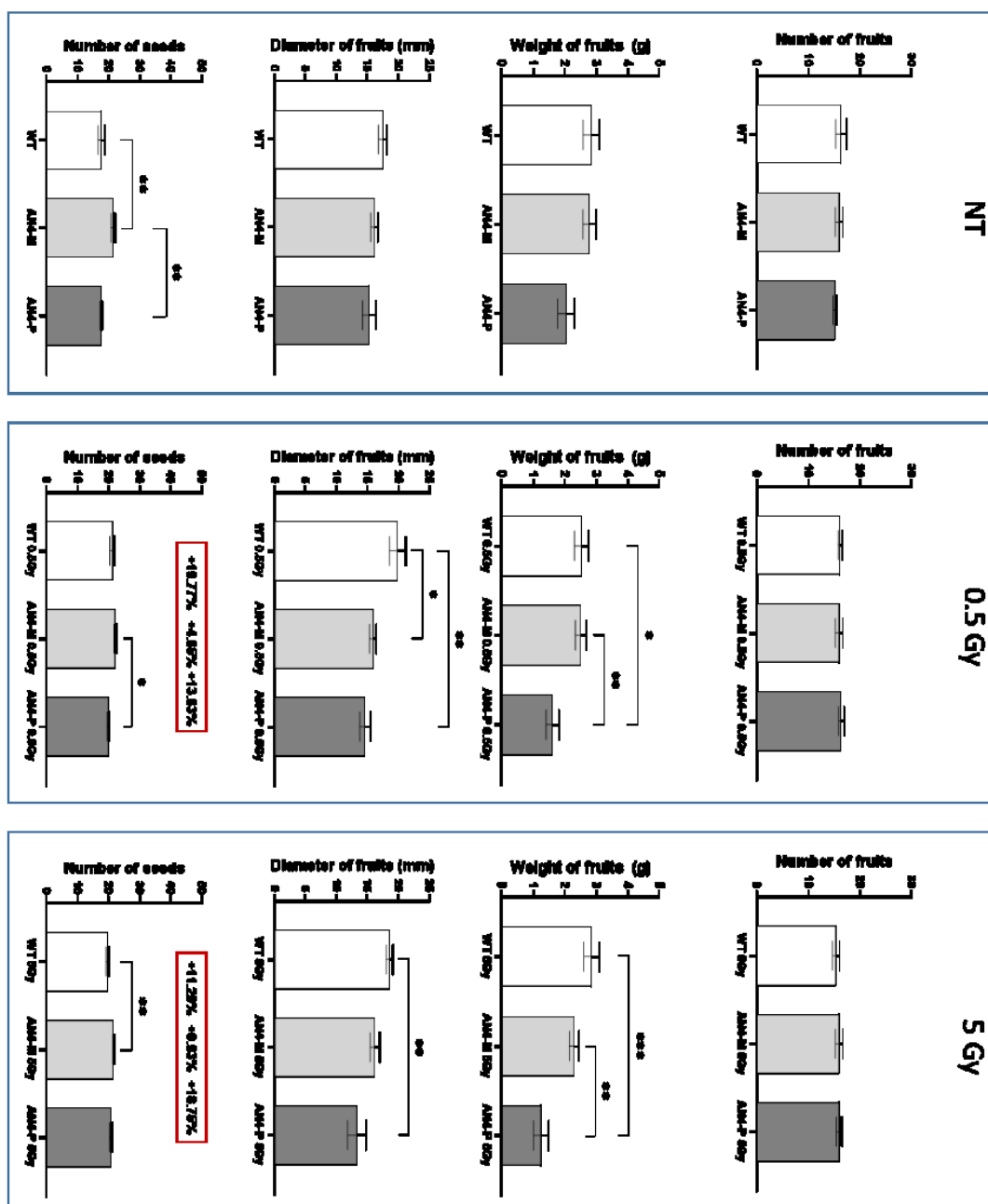


Figure 4. 53 Comparison of fruits-related parameters among the three genotypes according to the different treatments on plants irradiated at 30 DAS. doses (percentage increase compared to non-treated is shown in the red boxes).

Fluorimetric measurements related to SFR, FLAV, ANTH, NBI indices revealed that the irradiation of plants at 30 DAS produced several significant effects (Figure 4.54). Indeed, despite still non-significant, the interaction between the two variables ‘time’ and ‘gamma irradiation’ was calculated to be between 1.04 % and 31.5 %. The % of total variation due to the ‘gamma irradiation’ variable spaced between 1.16 % and 17.8 %, higher than that observed for irradiated seeds (from 0.28% to 6.63% as described in section 4.6.1), but still non relevant compared to total variation ascribed to ‘time’. In fact, the ‘time’ variable resulted between 27.3 % and 90.1 %.

	WT NT	WT 0.5Gy	WT 5Gy	WT 30Gy	
SFR	1.97±0.06	2.15±0.06	2.23±0.04	1.84±0.05	+ ***
FLAV	-0.15±0.01	-0.1±0.02	-0.12±0.006	-0.13±0.005	+ **
ANTH	-0.14±0.008	-0.12±0.009	-0.12±0.003	-0.11±0.001	+ *
NBI	1.9±0.08	1.99±0.01	2.16±0.04	1.86±0.02	/
					- *
					- **
					- ***
	AN4-M NT	AN4-M 0.5Gy	AN4-M 5Gy	AN4-M 30Gy	
SFR	1.91±0.07	2.06±0.05	1.8±0.05	1.39±0.05	
FLAV	-0.12±0.008	-0.11±0.004	-0.08±0.01	-0.05±0.009	
ANTH	-0.09±0.003	-0.09±0.003	-0.07±0.004	-0.07±0.004	
NBI	2±0.04	2.07±0.04	1.75±0.07	1.27±0.07	
	AN4-P NT	AN4-P 0.5Gy	AN4-P 5Gy	AN4-P 30Gy	
SFR	2.1±0.03	1.88±0.01	2.17±0.1	1.58±0.03	
FLAV	-0.13±0.01	-0.12±0.002	-0.1±0.005	-0.07±0.006	
ANTH	-0.05±0.004	-0.08±0.009	-0.06±0.001	-0.03±0.004	
NBI	2.2±0.12	2.16±0.14	2.29±0.15	1.61±0.02	

Figure 4. 54 Summary of fluorimetric results at the end of the measurement period analysed in comparison within each genotypes and according to the different treatments supplied to plants at 30 DAS. Data were subjected to two-way analysis of variance (two-way ANOVA) with Tukey’s post-hoc test to determine the differences in average of all tested parameters between irradiated and non-irradiated plants. NT: non-treated.

Data obtained at the end of the measurement period from wild type plants, only revealed a negative variation of ANTH and a positive variation of NBI index, both as a consequence of the 5 Gy dose supply. Interestingly, no variations of the indices were recorded after supplying the

30 Gy dose. In AN4-M plants, positive variations of the SFR (0.5 Gy dose), FLAV (5 and 30 Gy doses), ANTH (5 and 30 Gy doses), but negative variations of SFR (30 Gy) and NBI (5 and 30 Gy) indices were observed. In AN4-P₂ plants, positive variations were recorded for FLAV index at 30 Gy, while negative variations were observed for ANTH (0.5 Gy), SFR and NBI (30 Gy) indices.

Throughout the whole measurement period, the values detected for the SFR and NBI indices (Figures 4.55 and 4.56) progressively increased until 3 h AI (After Irradiation) as a prosecution of the data retrieved BI (Before Irradiation), for all the three genotypes and independently from the irradiation dose supplied. Thereafter, starting from 24 h A.I., a clear reset of the SFR index values on lower values progressively increasing until the end of measurements was recorded. In particular, at 3 h AI wild type plants subjected to the 0.5 Gy dose and AN4-P₂ plants subjected to the 5 Gy dose showed an increase of the SFR index while no changes occurred in AN4-M plants during the same period. Conversely, both AN4-M and AN4-P₂ plants showed statistically significant changes starting from 35 DAI (65 DAS) until the end of the detection period.

A periodic sigmoidal-like trend of values was observed for NBI, but with differences between the genotypes. In particular, the NBI value trend was positive until 3 h AI and decreased until 24 h AI and 3 DAI for wild type and AN4-M plants, respectively, to rise slightly and progressively until the end of measurements. In AN4-P₂ plants, NBI showed a periodic trend, as well, but with a higher frequency than wild type and AN4-M plants, with minimum at 10 min, 3 DAI (33 DAS) and 21 DAI (51 DAS), and maximum values BI, 3 h A.I. and 7 DAI (37 DAS). This trend was particularly significant for the AN4-P₂ plants that had received the 5 Gy dose, in which the NBI values significantly overwhelmed values recorded for the other doses (Figure 4.56). In addition, the 30 Gy dose had a significant negative effect on the NBI index of AN4-P₂ plants starting from 28 DAI (58 DAS) until the end of the detection period. This negative effect was also found in AN4-M plants starting from 21 DAI (51 DAS). Conversely, at 35 DAI (65 DAS) both 0.5 and 30 Gy caused a decrease of the NBI index in wild type plants. However, from 42 DAI (72 DAS) the 5 Gy dose had a positive effect on the NBI in wild type plants.

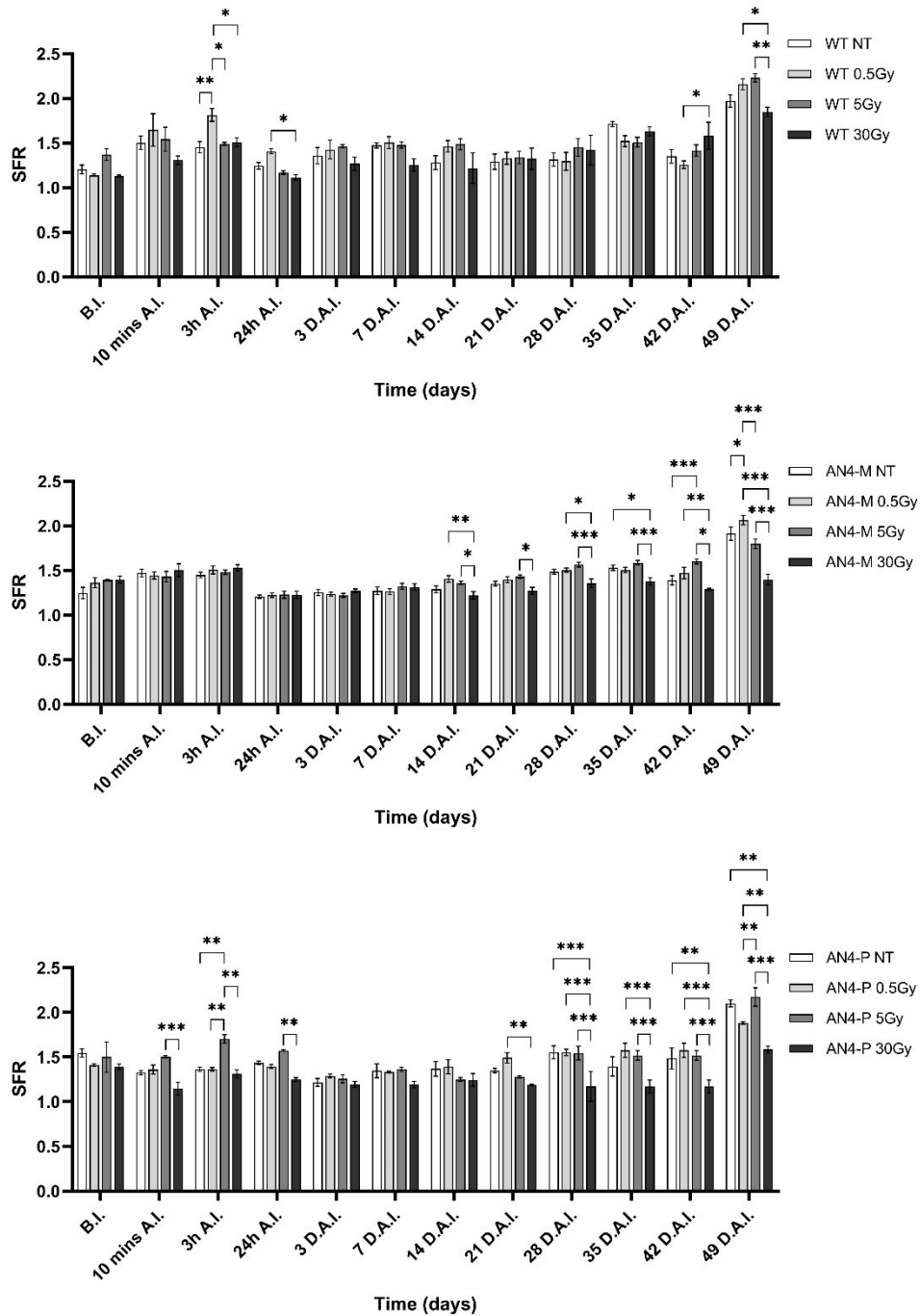


Figure 4. 55 Analysis of the SFR index of wild type, AN4-M and AN4-P2 MicroTom along the observation period upon different irradiation treatments supplied to plants at 30 DAS. Statistical relevance: *: $p < 0.05$; **: $p < 0.001$; ***: $p < 0.0001$.

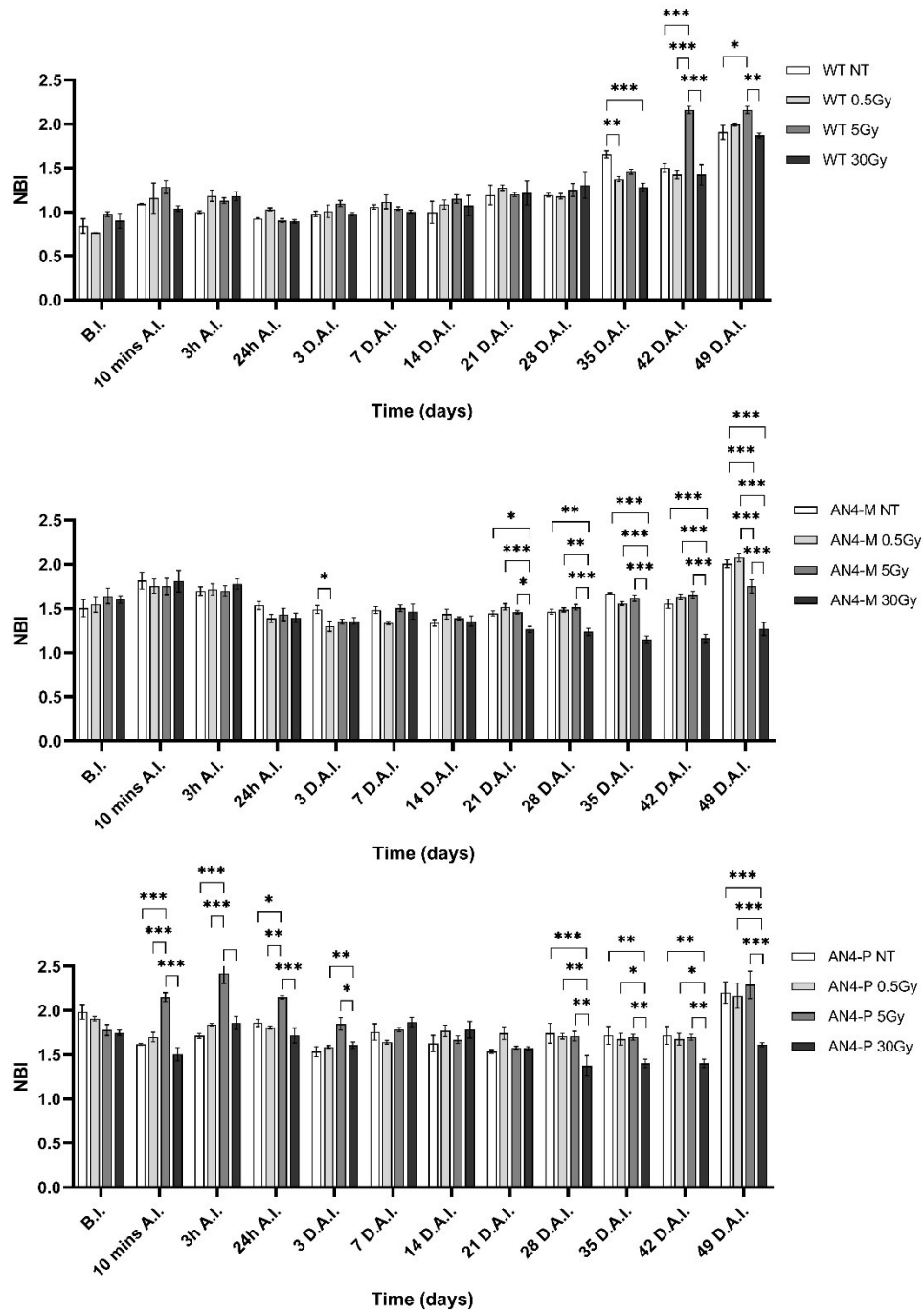


Figure 4. 56 Analysis of the NBI index of wild type, AN4-M and AN4-P₂ MicroTom along the observation period upon different irradiation treatments supplied at 30 DAS. Statistical relevance: *: $p < 0.05$; **: $p < 0.001$; ***: $p < 0.0001$.

The FLAV index values recorded throughout the measurement period showed a periodic sigmoidal-like shape trend only in wild type samples, with a value inversion point between 7 and 14 DAI (37-44 DAS) (Figure 4.57).

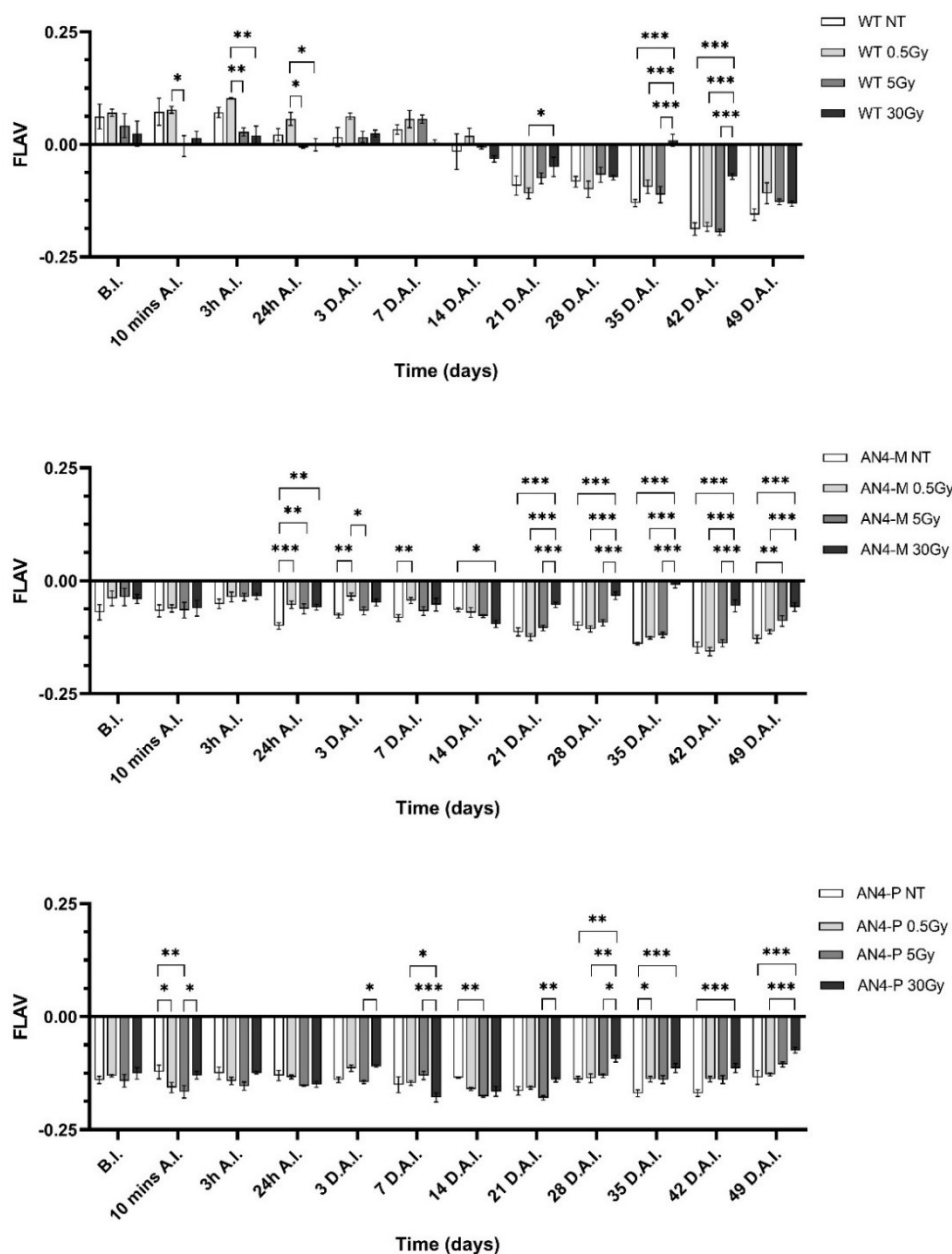


Figure 4. 57 Analysis of the FLAV index of wild type, AN4-M and AN4-P₂ MicroTom along the observation period upon different irradiation treatments supplied to plants at 30 DAS. Statistical relevance: *: $p < 0.05$; **: $p < 0.001$; ***: $p < 0.0001$.

This inversion is compatible with the metabolic changes related to the shift from the vegetative state to the flowering time, as explained for the offspring of irradiated seeds. On the contrary, the FLAV index values were registered as constantly negative from right BI (30 DAS) throughout life cycle of AN4 plants. This result probably reflects an anticipated decrease of flavonols levels

as precursors of anthocyanins biosynthesis. The 30 Gy dose supplied to wild type plants resulted in an increase of the FLAV index only at 35-42 (65-72 DAS). Conversely, AN4-M plants were positively affected by the irradiation starting from 24h AI until the end of measurements period for the 30 Gy dose, while the 0.5 and 5 Gy doses showed their major effect only during the first 3 DAI (33 DAS). Surprisingly the 0.5 and 5 Gy doses negatively affected AN4-P₂ plants straight after the irradiation (10 min AI) while the 30 Gy dose positively influenced the FLAV index of these plants starting at 28 DAI (58 DAS) until the end of the measurements.

As somehow expected and as seen in the experiment conducted on seeds, the ANTH index values defined differential curves for the wild type compared to the two transgenic genotypes (Figure 4.58). Indeed, while ANTH values were constant and negative throughout life cycle in wild type, indicating absence of anthocyanins, AN4 plants were characterized by a sigmoidal curve with a value inversion point between 7 and 14 DAI (corresponding to 37 and 44 DAS), corresponding to the flowering time. The 0.5 Gy dose supplied to wild type plants resulted in an increase of the ANTH index at 3-7 DAI (33-37 DAS) while the same index was positively influenced by the 30 Gy dose starting from 14 DAI (44 DAS) until the end of measurements period. Interestingly, in transgenic plants, variations of the ANTH index were recorded especially after this inversion point and mainly for the 30 Gy dose (for AN4-P₂ plants, some differences started at 3 DAI).

In general, the decrease of the indices correlated to the flavonoids and secondary metabolism (FLAV and ANTH) in the second half of life cycle matches with the increase of SFR and NBI (indices of the primary metabolism), as expected.

Lastly, as previously showed for measurements conducted on irradiated seeds, the photosynthetic maximum photochemical efficiency (Fv/Fm) and the maximum fluorescent yield (Y_Fm) always resulted in the range described by the Plant Stress Kit (PSKII) manufacturer (0.7/0.9 for healthy leaves) but some statistically differences were noticed. In particular, Fv/Fm resulted in a significant decrease in wild type plants treated with the 30 Gy dose starting from 10 minutes after irradiation followed by a slight increase during the first 7 days (Figure 4.59). No statistical differences were noticed for transgenic plants.

Regarding the Y_Fm index, no changes were found at the end of the measurements period (Figure 4.60). However, the 5 and 30 Gy dose supplied to wild type resulted in a slight increase of the Y_Fm index straight after the irradiation (10 min AI). Conversely, 10 minutes after irradiation a slight decrease was found in AN4-P₂ plants subjected to 0.5 and 5 Gy doses while until 24h AI some significant differences were found also for plants subjected to the 30 Gy dose. In summary, the fluorimetric indices undergo some variations in dependence of the genotype and treatment considered. As previously reported for irradiated seeds, also in this case no significant

changes occurred at the photosynthetic apparatus, demonstrating that, in our experimental conditions, gamma rays do not affect the photosynthetic performances.

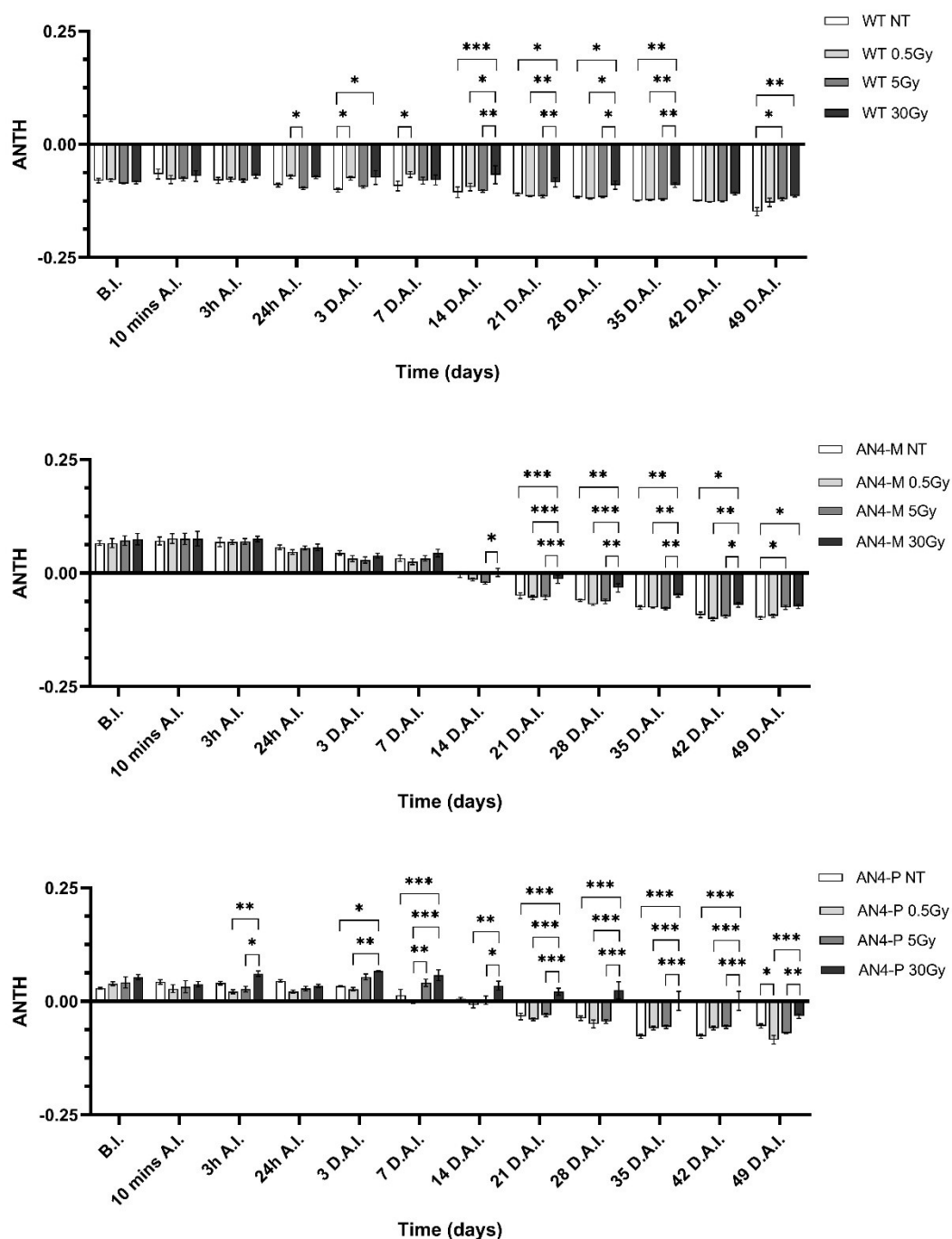


Figure 4. 58 Analysis of the ANTH index of wild type, AN4-M and AN4-P₂ MicroTom along the observation period upon different irradiation treatments supplied to plants at 30 DAS. Statistical relevance: *: $p < 0.05$; **: $p < 0.001$; ***: $p < 0.0001$.

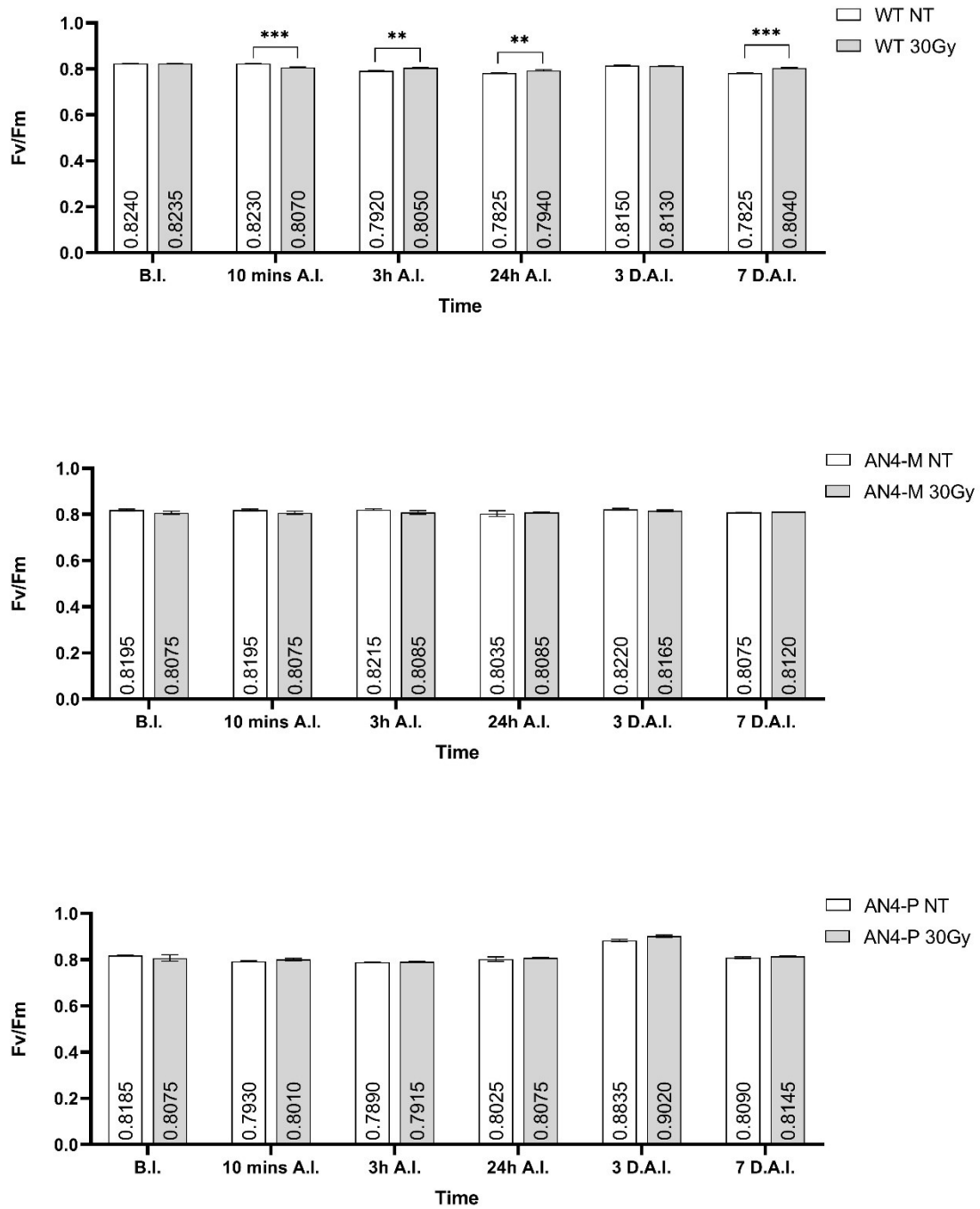


Figure 4. 59 Analysis of the Fv / Fm index of wild type, AN4-M and AN4-P₂ MicroTom along the observation period upon different irradiation treatments supplied to plants at 30 DAS. Statistical relevance: *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.0001$.

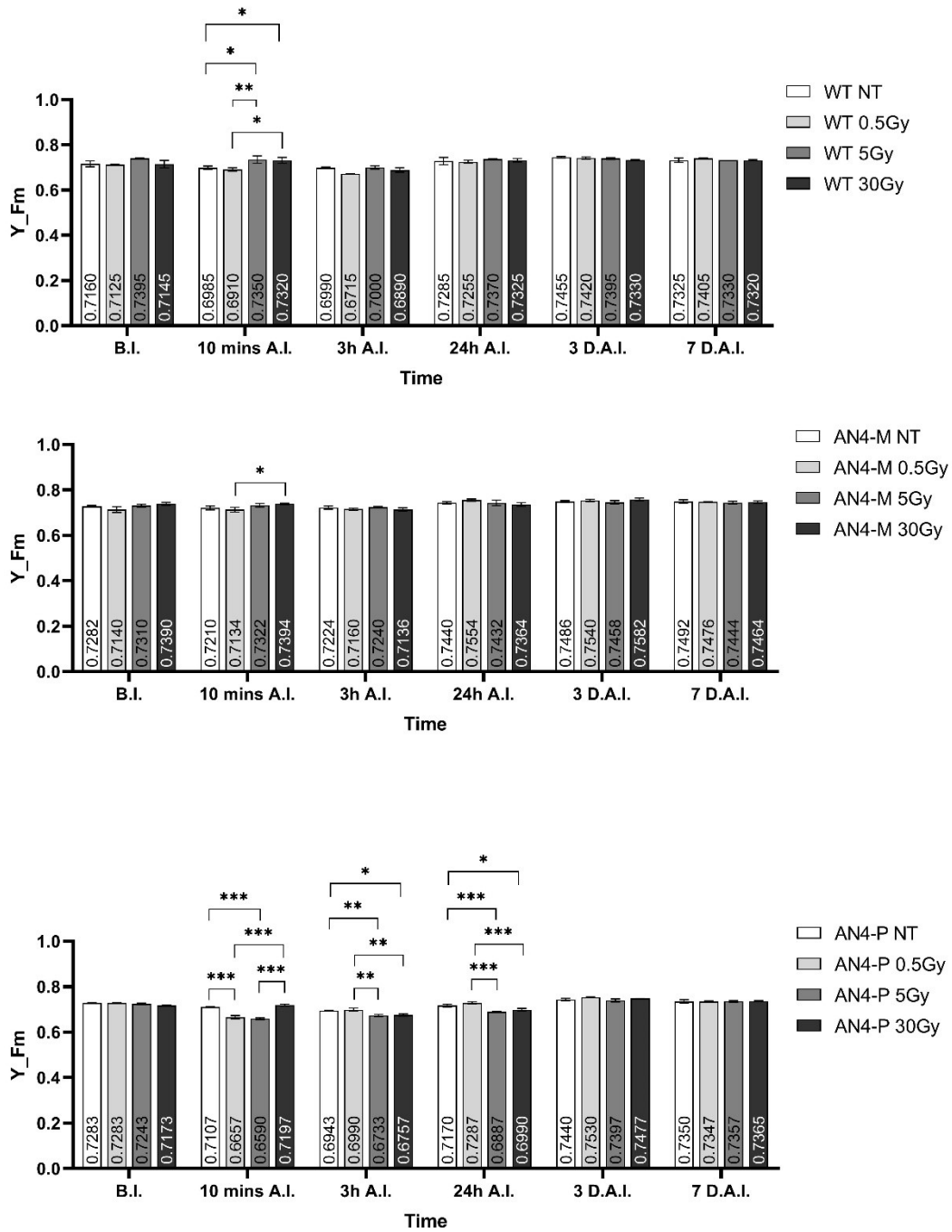


Figure 4. 60 Analysis of the Y_Fm index of wild type, AN4-M and AN4-P₂ MicroTom along the observation period upon different irradiation treatments supplied to plants at 30 DAS. Statistical relevance: *: $p < 0.05$; **: $p < 0.001$; ***: $p < 0.0001$.

4.6.2.1 Biometric and fluorimetric characterization of the second generation of plants irradiated at 30 DAS

As reported by Kordyum and Hasenstein, 2021, little information are available about the inherited effects of IR on subsequent generations of plants. Plants that had been irradiated with 0.5 and 5 Gy at 30 DAS were able to produce viable seeds, and, therefore, the offspring of these plants were subjected to biometric measurements at three time points (30, 60 and 90 DAS). Irradiation with 30 Gy applied to plants at 30 DAS had determined no seed production, therefore no evaluations could be carried on. A PCA analysis was performed (Figure 4.61). The contributions of the first two PCs were 35.17% (PC1) and 16.65% (PC2), respectively, and their sum explained the 51.82% of the phenotypic variation. The loadings plot (Figure 4.61A) shows that FLAV, ANTH and leaf area and number of seeds are positively correlated with the PC1, while the number of leaves, number of flowers, number of fruits, height, leaf area, FLAV and ANTH are positively correlated with the PC2. SFR, NBI and the weight of fruits resulted negatively correlated with both PC1 and PC2, while leaf area, FLAV and ANTH resulted positively correlated with both PC1 and PC2. The score plot and the biplot (Figure 4.61B and Figure 4.61C) showed a less specific localization of outcomes in function of the three different genotypes compared to the experiments conducted on the parental plants. The scores of wild type plants still clustered in the left quadrants of the plot (negative variations of PC1). In particular, non-treated wild type plants still showed negative correlation with both PC1 and PC2, while irradiated wild type plants, irrespective of doses, were found in the upper left quadrant (negative correlation with PC1 and positive with PC2). Scores related to AN4-M clustered near the y axes, demonstrating greater contribution to the overall variation of PC1 and PC2 compared to the parental plants. The scores of the AN4-P₂ plants still clustered in the upper-right and lower-right corner (positive correlation with PC1 and positive or negative correlation with PC2).

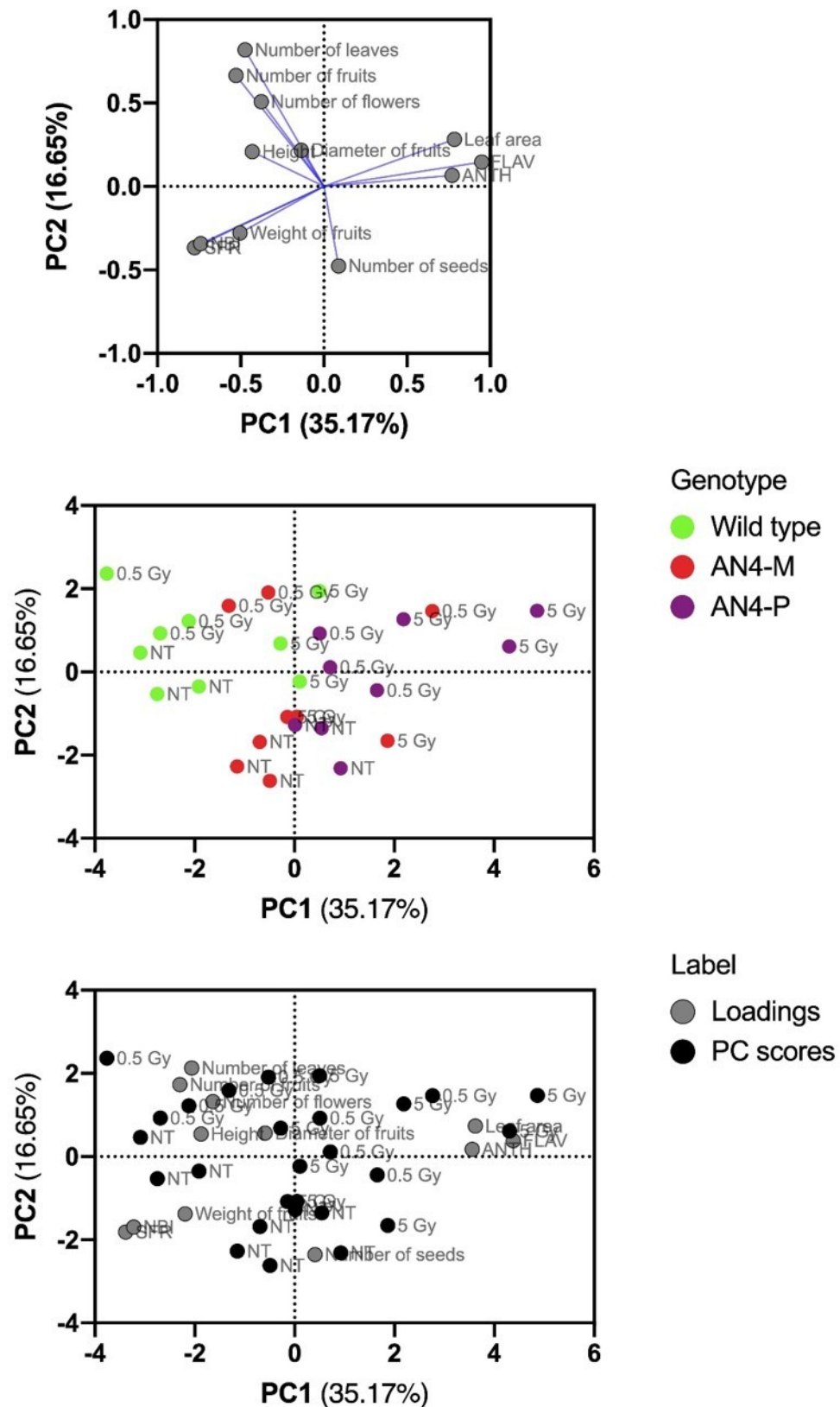


Figure 4. 61 Principal component analysis on morphometric and fluorimetric parameters obtained from data measured on offspring of plants irradiated at 30 DAS. Results are graphically reported using the Loadings plot with all the parameters considered (A), the PC scores plot with wild type and AN4 plants (B) and the biplot that contains the information presented in both the Loadings plot and the PC scores plot at the same time (C).

From the analysis of variance (two-way Anova), as summarized in Figure 4.62, the parental irradiation with 0.5 and 5 Gy doses was able to exert a positive influence on some morphometric parameters of the offspring. Specifically, the non-significant interaction between ‘time’ and ‘treatment’ variables did not exceed the 18.5% of the total variation. The % of total variation ascribed to the ‘gamma irradiation’ variable always resulted included between 1.02% and 33.2%, much higher than that observed on irradiated seeds and plants irradiated at 30 DAS (0.002-2.31% and 1.21-22%, respectively), revealing a certain effect of parental radiation onto offspring. The % of variation ascribed to the ‘time’ variable was included between 36.4% and 89.3%, indicating a greater effect of the variable ‘time’ compared to the variable ‘gamma irradiation’.

	WT NT	WT 0.5Gy	WT 5Gy	
Height (cm)	18.66±2.33	20±0.57	20±0.57	+ ***
Number of leaves	25.66±3.17	32±4.35	24±2.08	+ **
Leaf area (mm ²)	76.94±8.9	85.11±3.42	115.88±18.31	+ *
Number of flowers	4±2.08	3.33±0.66	1.66±1.66	/
Number of fruits	14.33±1.2	20.66±2.96	12.66±0.33	- *
Weight of fruits (g)	3.08±0.24	2.8±0.2	2.78±0.18	- **
Diameter of fruits (mm)	17.41±0.48	18.19±0.67	18.13±0.43	- ***
Number of seeds	20±0.53	17.73±0.89	19.6±0.6	

	AN4-M NT	AN4-M 0.5Gy	AN4-M 5Gy	
Height (cm)	18.66±1.76	18.66±1.2	16.33±0.88	
Number of leaves	9.66±1.45	28.66±3.84	13±1	
Leaf area (mm ²)	71.17±29.24	110.51±21.53	142.01±6.49	
Number of flowers	1±0.57	4±2.64	1.66±0.33	
Number of fruits	8.66±1.45	15.66±1.45	11.33±1.66	
Weight of fruits (g)	2.47±0.17	2.26±0.2	2.55±0.17	
Diameter of fruits (mm)	16.24±0.43	16.39±0.41	16.64±0.57	
Number of seeds	24.33±0.89	21.6±0.4	21.46±1.09	

	AN4-P NT	AN4-P 0.5Gy	AN4-P 5Gy	
Height (cm)	15±1.73	16.33±1.45	15±1.15	
Number of leaves	14±1	17.66±2.84	18±1.73	
Leaf area (mm ²)	118.66±14.35	142.89±7.95	210.87±20.84	
Number of flowers	/	1.66±1.2	2±1.15	
Number of fruits	9.66±1.45	15±3.51	10.66±0.88	
Weight of fruits (g)	2.24±0.23	1.75±0.18	1.92±0.15	
Diameter of fruits (mm)	15.68±0.72	15.38±0.66	14.72±1.11	
Number of seeds	18.66±0.47	16.6±0.86	18.6±0.6	

Figure 4. 62 Summary of morphometric results at the end of the measurements period analysed in comparison within each genotypes and according to the different treatments on descendants of plants irradiated at 30 DAS. Data were subjected to two-way analysis of variance (two-way ANOVA) with Tukey’s post-hoc test to determine the differences in average of all tested parameters between irradiated and non-irradiated plants. NT: non-treated.

At the end of the measurement period, interestingly, the leaf area resulted in a statistically significant increase for the offspring of all the genotypes (wild type, AN4-M and AN4-P₂ plants) derived from plants treated with the 5 Gy dose. Further variations were observed only in descendants of AN4-M plants treated with the 0.5 Gy dose, and consisted in statistically significant increase of the number of leaves and of the number of fruits (Figure 4.62). It is interesting to note that, for both 0.5 and 5 Gy dose, no detrimental effects were detected on other yield traits such as number of flowers, diameter of fruits, weight of fruits, number of seeds (Figure 4.62 and Figures from 4.63 to 4.70).

Following the traits along the whole observational period, regardless of possible maintenance of the variation until the end of the measurements, the parental treatment with 0.5 Gy and 5 Gy initiated the positive effects before the 90 DAS. Wild type and AN4-M plants showed a significant increase of number of leaves and number of flowers already at 60 DAS but only in offspring of AN4-M plants irradiated at 0.5 Gy was found an increase of number of fruits. Surprisingly, no one of the statistically significant changes that have been found are the same of that encountered on the parental lines.

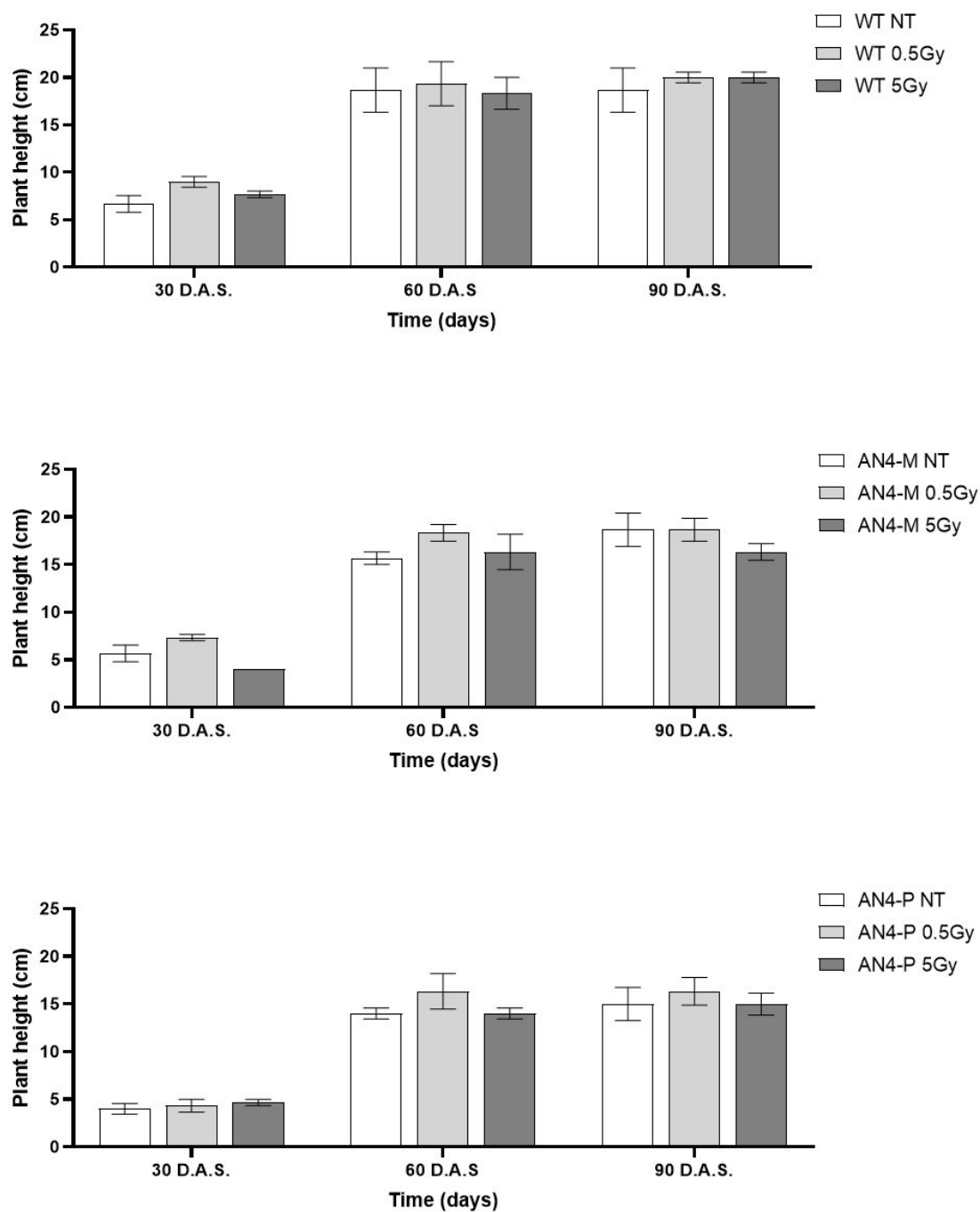


Figure 4. 63 Analysis of plant height of wild type, AN4-M and AN4-P₂ MicroTom along the observation period and conducted on the offspring obtained from plants irradiated at 30 DAS. Statistical relevance: *: $p < 0.05$; **: $p < 0.001$; ***: $p < 0.0001$.

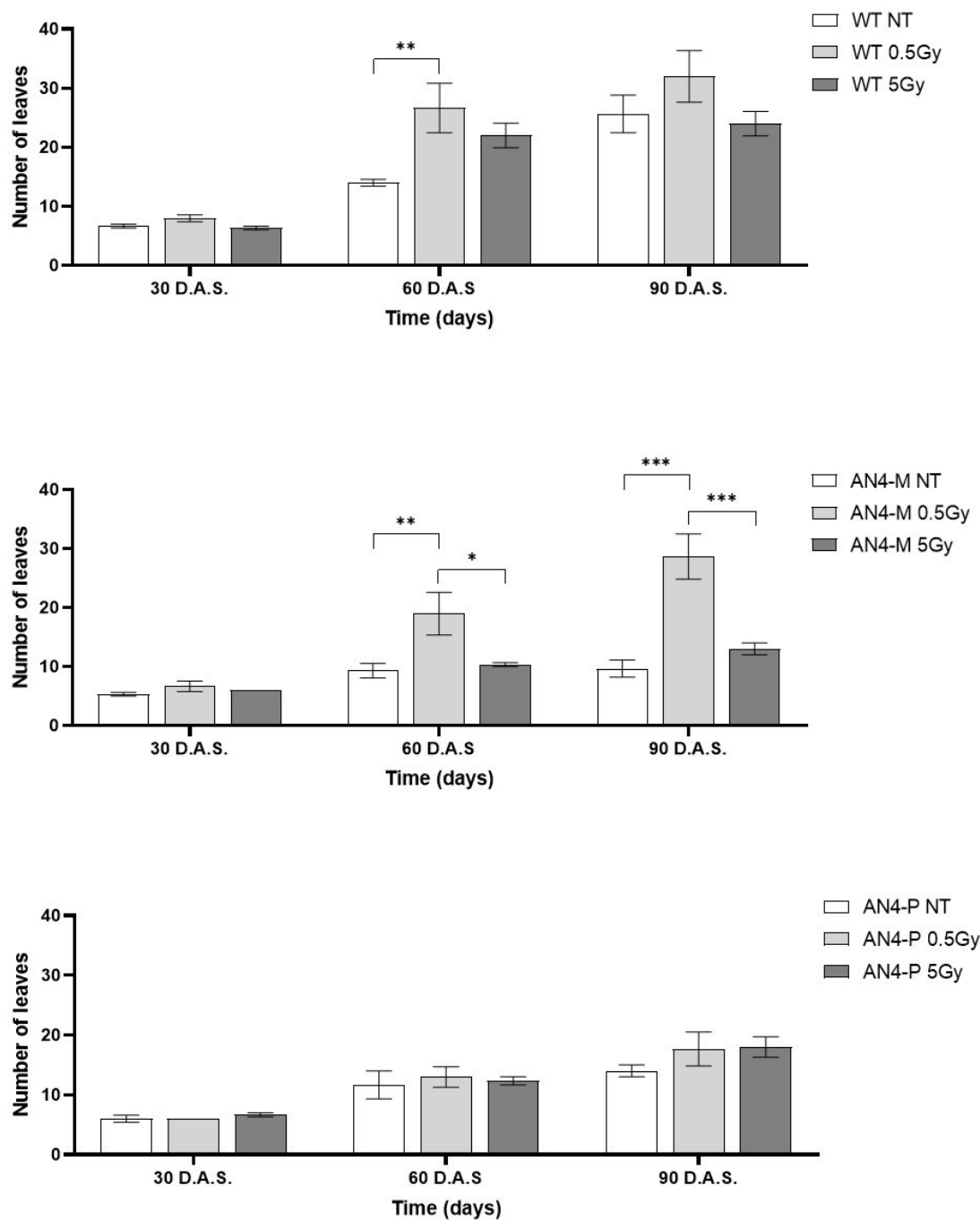


Figure 4. 64 Analysis of number of leaves of wild type, AN4-M and AN4- P₂ MicroTom plants along the observation period and conducted on the offspring obtained from plants irradiated at 30 DAS. Statistical relevance: *: $p < 0.05$; **: $p < 0.001$; ***: $p < 0.0001$.

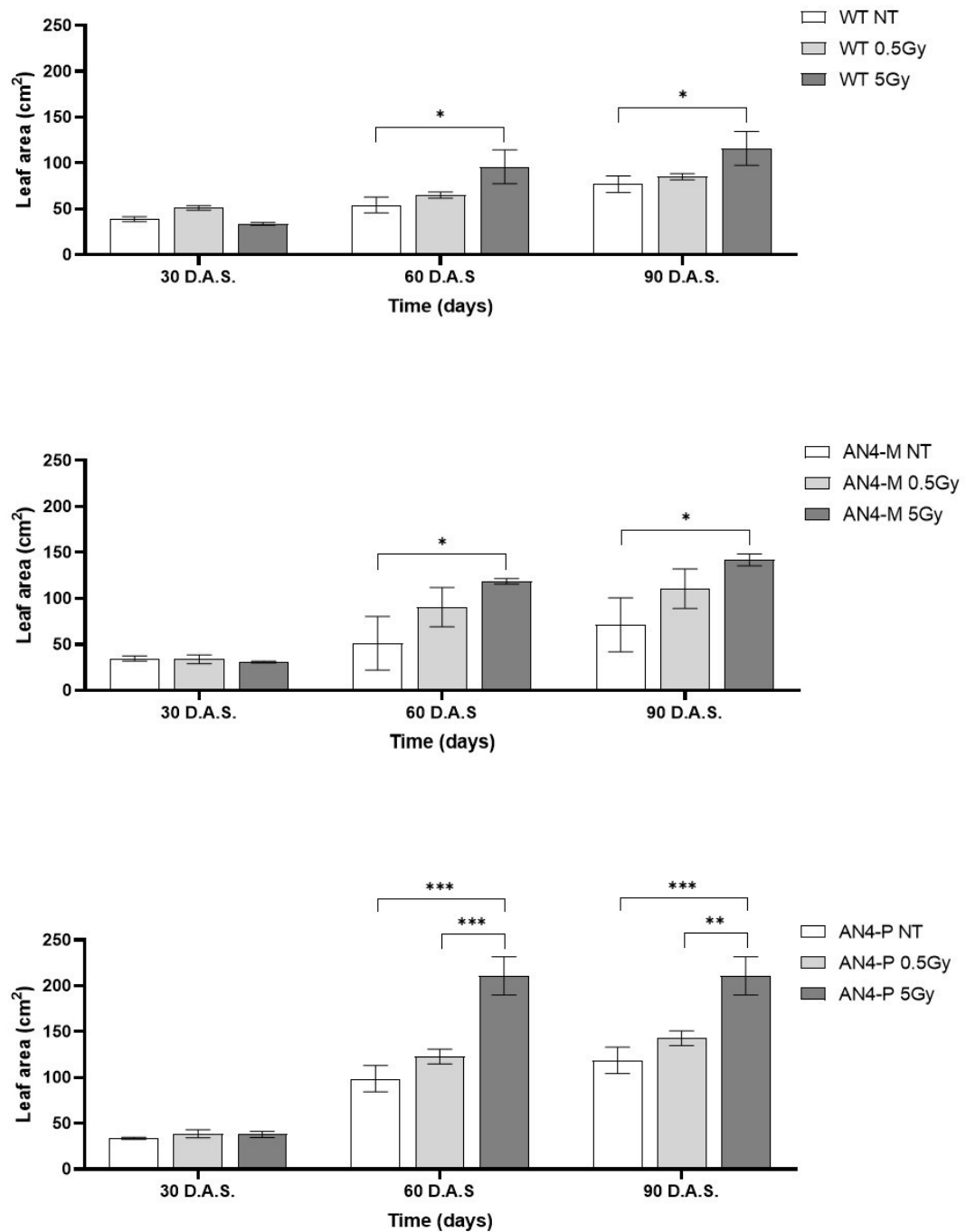


Figure 4.65 Analysis of leaf area of wild type, AN4-M and AN4-P₂ MicroTom plants along the observation period and conducted on the offspring obtained from plants irradiated at 30 DAS. Statistical relevance: *: $p < 0.05$; **: $p < 0.001$; ***: $p < 0.0001$.

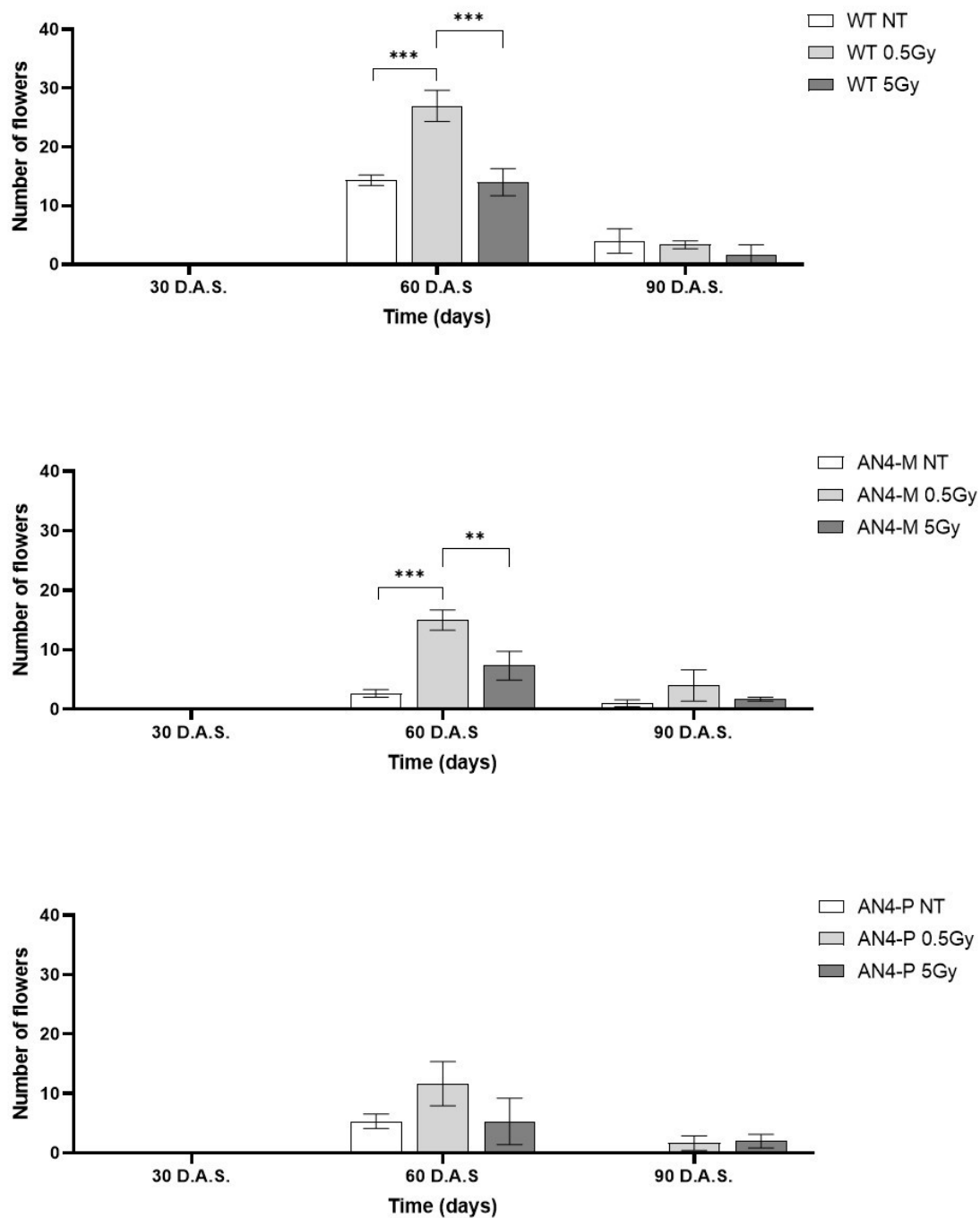


Figure 4. 66 Analysis of number of flowers of wild type, AN4-M and AN4-P₂ MicroTom plants along the observation period and conducted on the offspring obtained from plants irradiated at 30 DAS. Statistical relevance: *: $p < 0.05$; **: $p < 0.001$; ***: $p < 0.0001$.

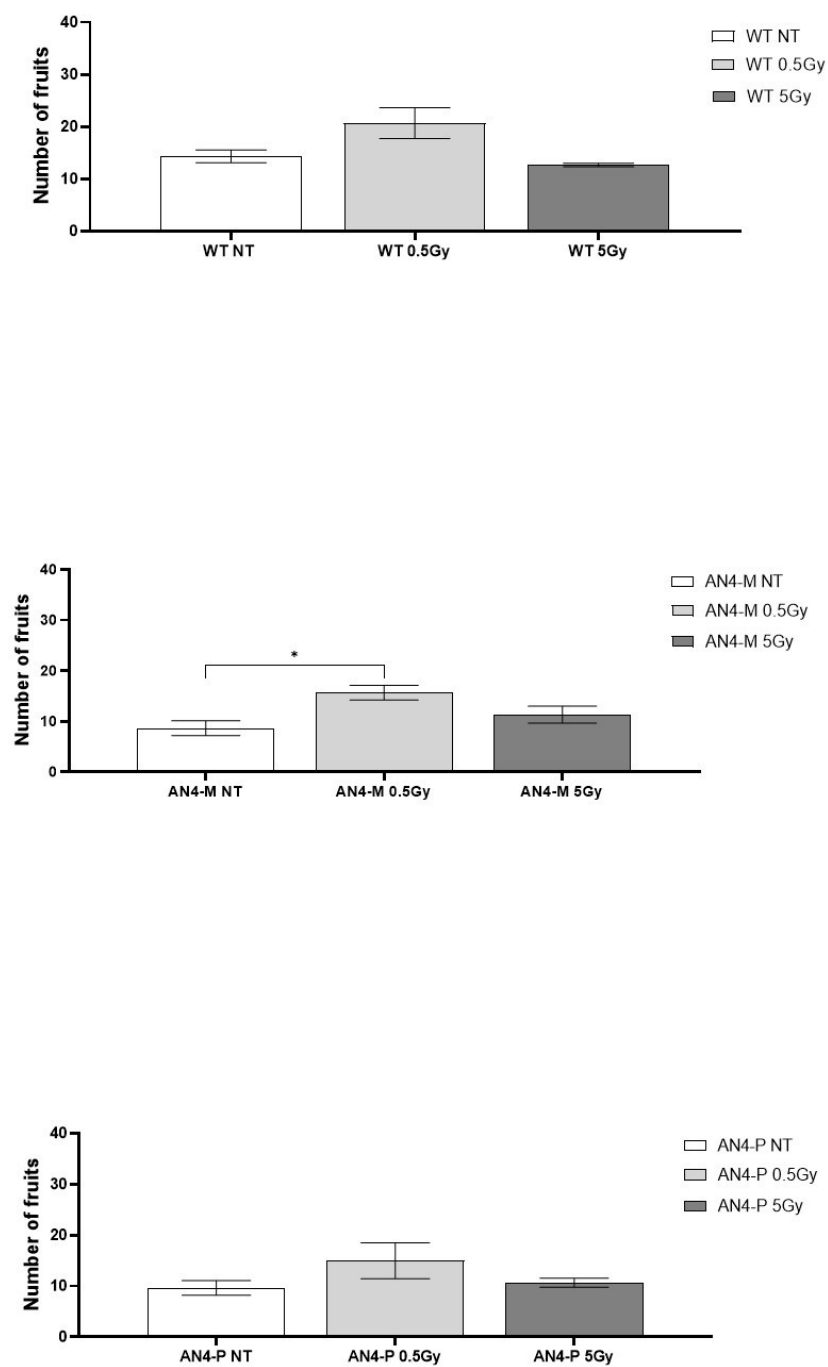


Figure 4. 67 Analysis of number of fruits of wild type, AN4-M and AN4-P₂ MicroTom plants at the end of the life cycle conducted on the offspring obtained from plants irradiated at 30 DAS. Statistical relevance: *: $p < 0.05$; **: $p < 0.001$; ***: $p < 0.0001$.

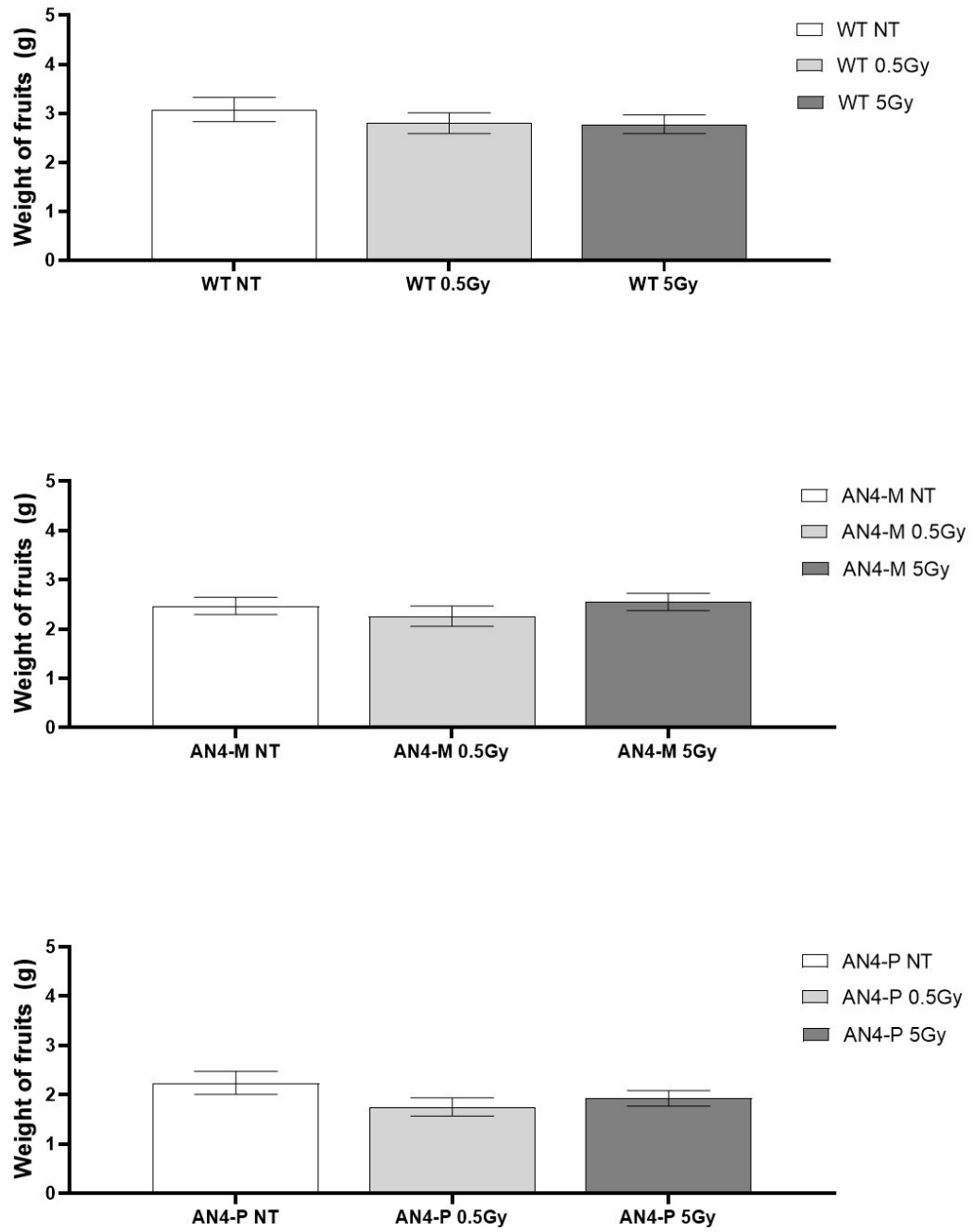


Figure 4. 68 Analysis of weight of fruits of wild type, AN4-M and AN4-P₂ MicroTom plants at the end of the life cycle conducted on the offspring obtained from plants irradiated at 30 DAS. Statistical relevance: *: $p < 0.05$; **: $p < 0.001$; ***: $p < 0.0001$.

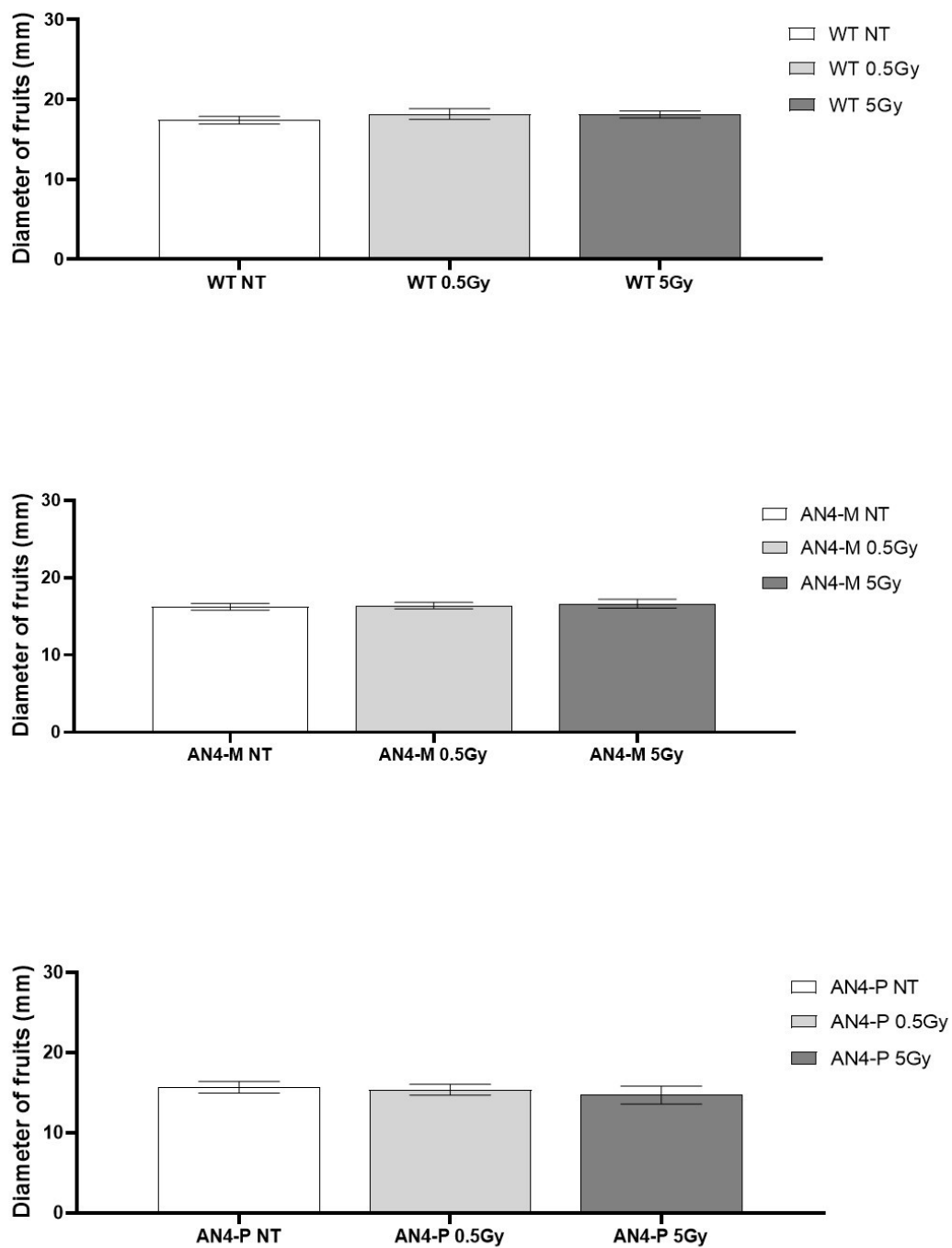


Figure 4. 69 Analysis of diameter of fruits of wild type, AN4-M and AN4-P₂ MicroTom plants at the end of the life cycle conducted on the offspring obtained from plants irradiated at 30 DAS. Statistical relevance: *: $p < 0.05$; **: $p < 0.001$; ***: $p < 0.0001$.

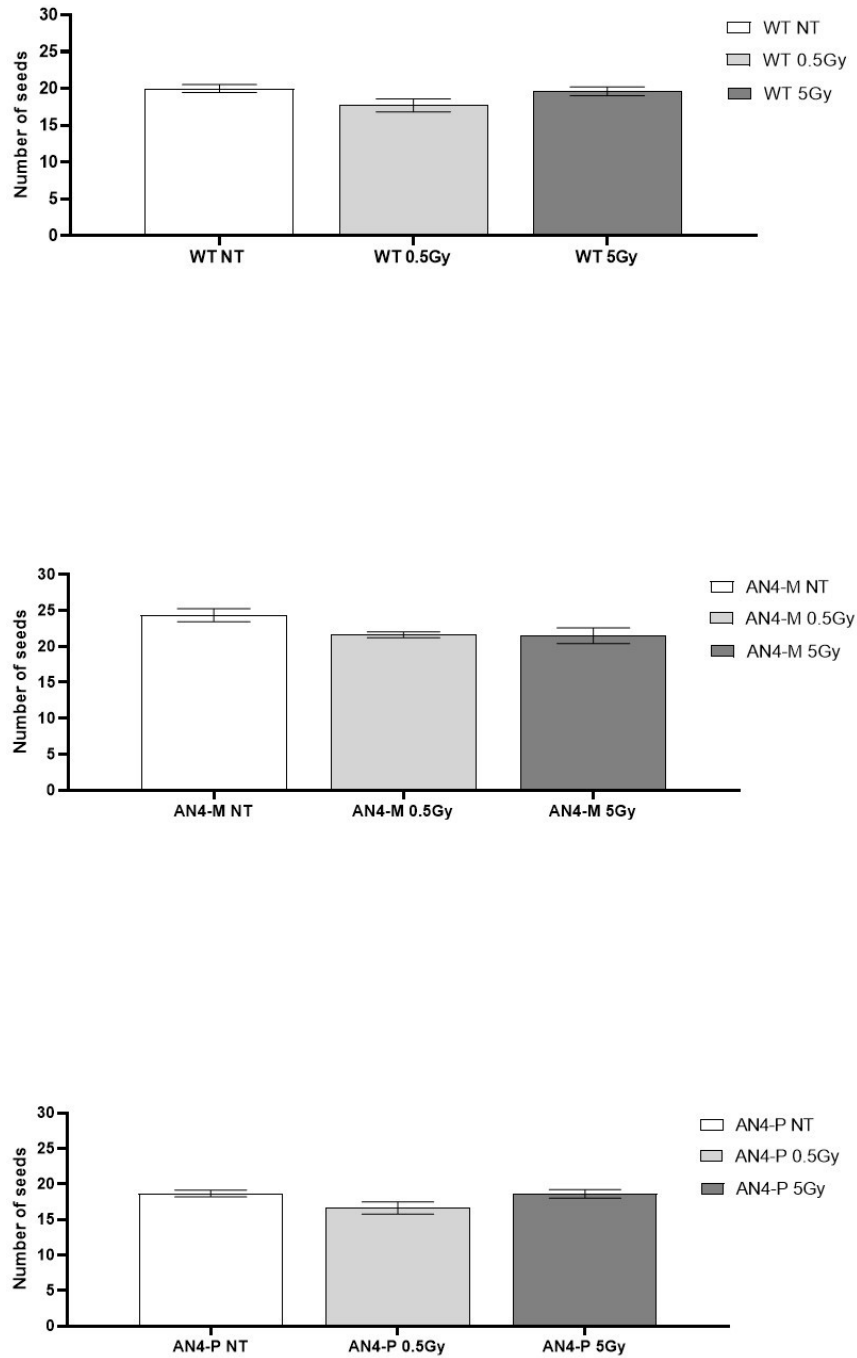


Figure 4. 70 Analysis of number of seeds of wild type, AN4-M and AN4-P2 MicroTom fruits conducted on the offspring obtained from plants irradiated at 30 DAS. Statistical relevance: *: $p < 0.05$; **: $p < 0.001$; ***: $p < 0.0001$.

The values of fruits-related parameters at fruit set, were compared among the three genotypes in function of the radiation dose, as well (Figure 4.71) and, in accordance with data obtained from from irradiated seeds and plants irradiated at 30 DAS, no statistical variation was observed in number of fruits. Conversely, 0.5 and 5 Gy doses negatively affected the weight and diameter of fruits of AN4-P₂ plants. On the contrary, the three genotypes are significantly different for

number of seeds already in standard conditions. They remain different also upon radiation, with low doses (0.5 and 5 Gy) determining a decrease of the number of seeds in all the three genotypes.

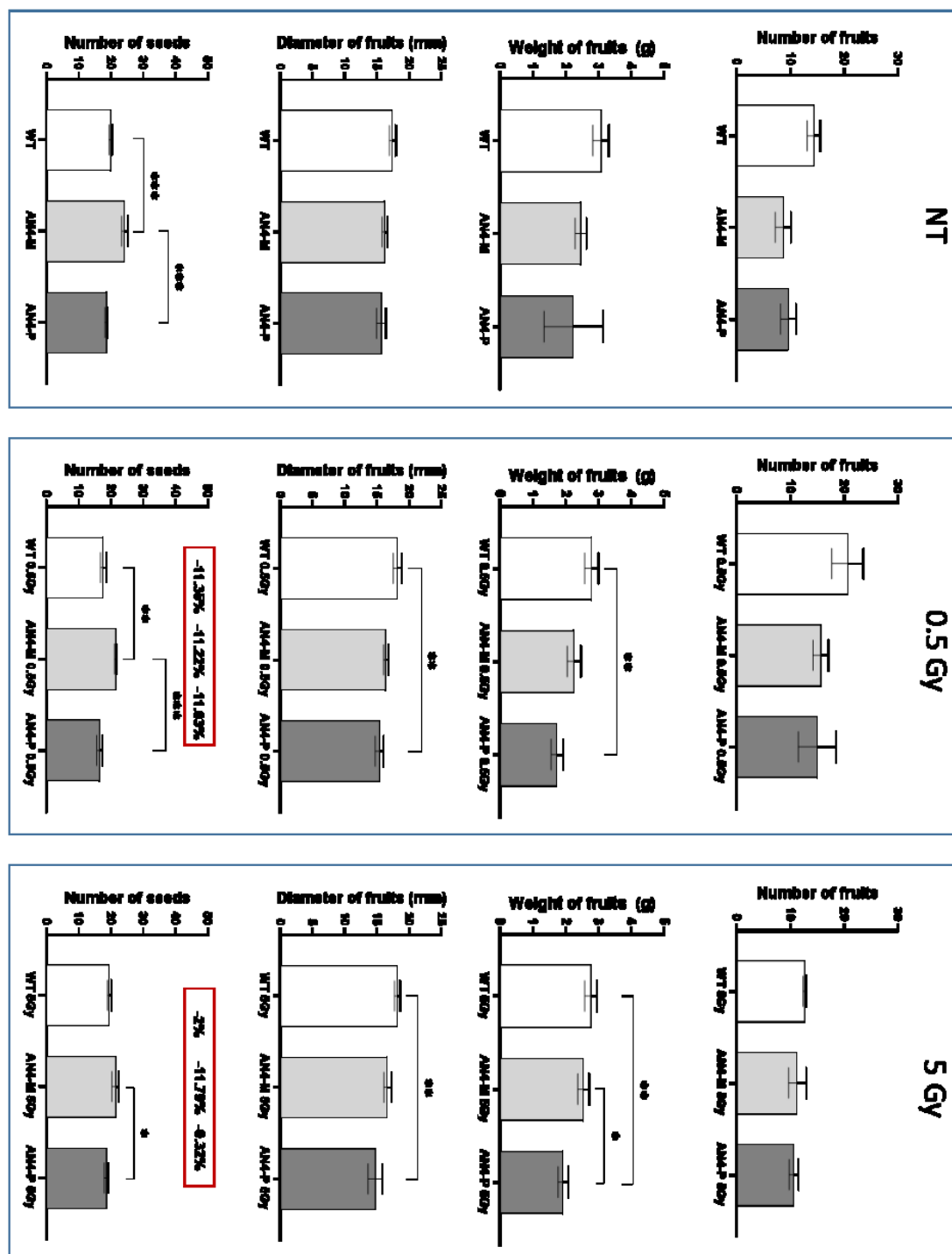


Figure 4. 71 Comparison of fruits-related parameters on offspring of plants irradiated at 30 DAS.

As summarized in Figure 4.72, several differences were found on fluorimetric indices measured by Multiplex Force A™. The % of total variation due to the ‘gamma irradiation’ variable spaced between 0.39% and 15.5% (similar to that observed in parental plants but higher than that

observed for irradiated seeds), while the ‘time’ variable resulted between 1.68% and 87.6%. The not significant interaction between the two variables was calculated to be between 1.4% and 30.5% indicating a greater effect of the variable ‘time’ compared to the variable ‘gamma irradiation’.

At the end of the measurements period, the SFR index showed a significant decrease in descendants of both wild type and AN4-P₂ plants that had been previously subjected to a 5 Gy dose and an increase in AN4-M descendants derived from plants irradiated with the same dose.

	WT NT	WT 0.5Gy	WT 5Gy		
SFR	1.54±0.08	1.62±0.019	1.3±0.04	+	***
FLAV	-0.15±0.006	-0.14±0.005	-0.09±0.01	+	**
ANTH	-0.14±0.007	-0.13±0.008	-0.12±0.004	+	*
NBI	1.48±0.06	1.56±0.03	1.2±0.07	/	
				-	*
				-	**
				-	***
	AN4-M NT	AN4-M 0.5Gy	AN4-M 5Gy		
SFR	1.34±0.1	1.13±0.05	1.44±0.08		
FLAV	-0.13±0.003	-0.05±0.02	-0.06±0.01		
ANTH	-0.13±0.01	-0.08±0.01	-0.08±0.005		
NBI	1.28±0.12	1.04±0.04	1.35±0.09		
	AN4-P NT	AN4-P 0.5Gy	AN4-P 5Gy		
SFR	1.51±0.02	1.38±0.04	1.11±0.13		
FLAV	-0.08±0.007	-0.05±0.008	0.001±0.01		
ANTH	-0.08±0.007	-0.06±0.005	-0.08±0.01		
NBI	1.5±0.02	1.33±0.07	0.95±0.08		

Figure 4. 72 Summary of fluorimetric results at the end of the measurements period analysed in comparison within each genotype and according to the different treatments on descendants of plants irradiated at 30 DAS. Data were subjected to two-way analysis of variance (two-way ANOVA) with Tukey’s post-hoc test to determine the differences in average of all tested parameters between irradiated and non-irradiated plants. NT: non-treated.

With the only exception of individuals derived from wild type plants irradiated with the 0.5 Gy dose, all the genotypes showed a significant increase in the FLAV index. The ANTH index showed an increase only in individuals derived from AN4-M plants subjected to both 0.5 and 5 Gy doses, while NBI was negatively affected in individuals derived from both wild type and AN4-P₂ plants treated with the 5 Gy dose and from AN4-M plants treated with the 0.5 Gy dose. Looking at the overall measurement period, the variations mainly came out at the end of the measurement period for SFR and NBI (with the only exception of a negative additional variation

of NBI in descendants of AN4-P₂ plants irradiated at 0.5 Gy that was recorded at 30 DAS and a negative variation at 5 Gy in AN4-M plants at 60 DAS) (Figure 4.73 and Figure 4.74).

The FLAV index values recorded throughout the measurement period showed a sigmoidal-like shape trend in wild type and AN4-M descendants, with a value inversion point between 30 and 60 DAS (similar to that of 37-44 DAS seen for the parental plants) (Figure 4.75). On the contrary, the FLAV index values were registered as constantly negative for AN4-P₂ plants. In all the three genotypes the irradiation of the parental plants resulted in higher values of the FLAV index starting from 30 DAS, especially in the AN4 offspring.

The ANTH index values defined differential curves for the wild type compared to the two transgenic genotypes (Figure 4.76). Indeed, while ANTH values were constant and negative throughout life cycle in wild type descendants, indicating absence of anthocyanins, both AN4-M and AN4-P₂ offspring were characterized by a sigmoidal curve with a value inversion point between 30 and 60 DAS (similar to that of 37 and 44 DAS of the parental plants). Only in AN4-P₂ plants, the irradiation of parental lines resulted in a visible effect at 60 DAS, with the values that returned stable at the end of the measurements.

Finally, both the Fv/Fm and the Y_Fm indices resulted in the range described by the Plant Stress Kit (PSKII) manufacturer during all the measurements period, independently from the genotype and from the irradiation procedure that were subjected to (Figure 4.77 and Figure 4.78).

All together, these results demonstrated that some changes occurred to the offspring of plants irradiated at 30 DAS. Surprisingly, the significantly different parameters were not the same detected in the parental genotypes with the only exception of FLAV and ANTH indices in AN4-M plants subjected to the 5 Gy dose.

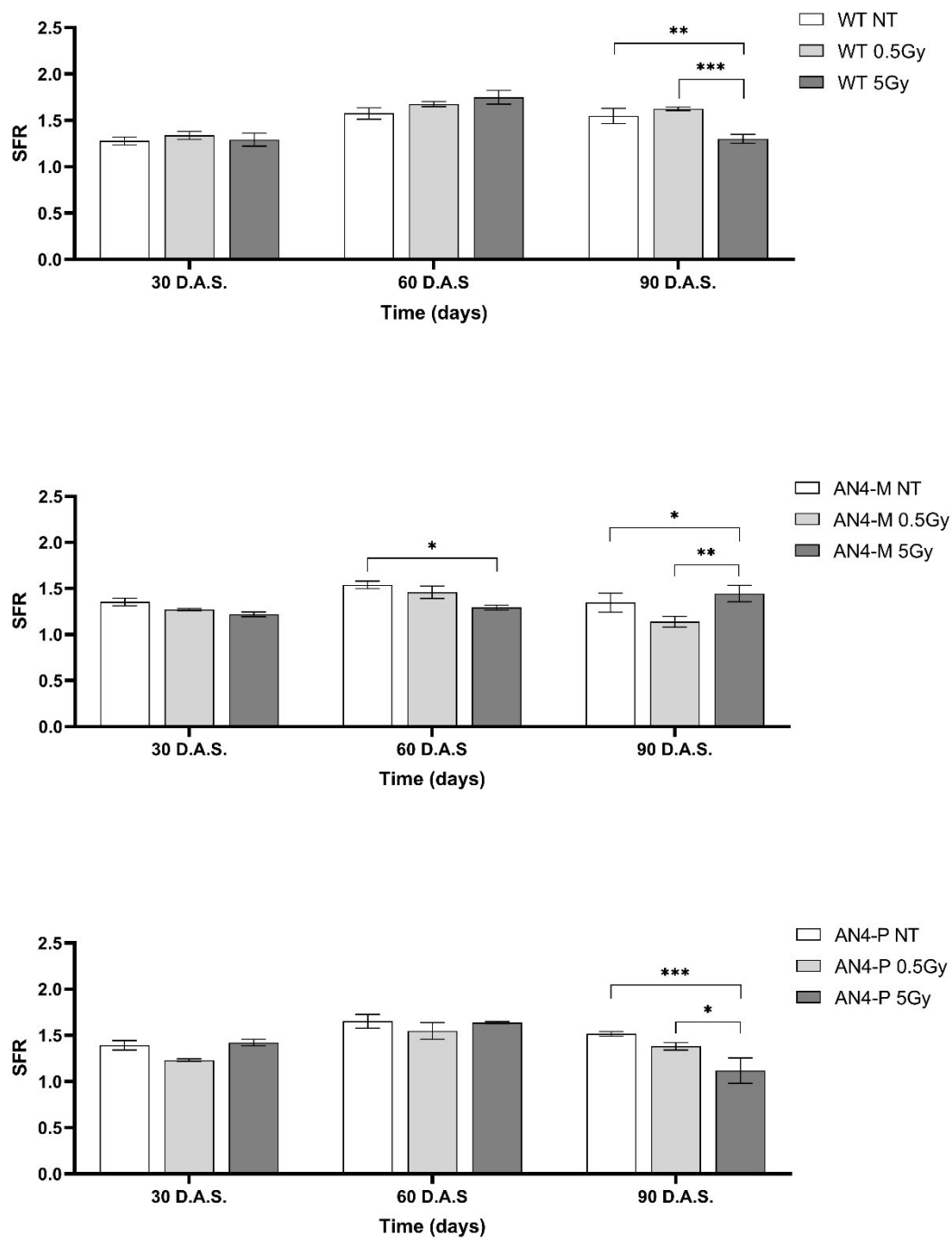


Figure 4. 73 Analysis of the SFR index of wild type, AN4-M and AN4-P₂ MicroTom along the observation period conducted on the offspring obtained from plants irradiated at 30 DAS. Statistical relevance: *: $p < 0.05$; **: $p < 0.001$; ***: $p < 0.0001$.

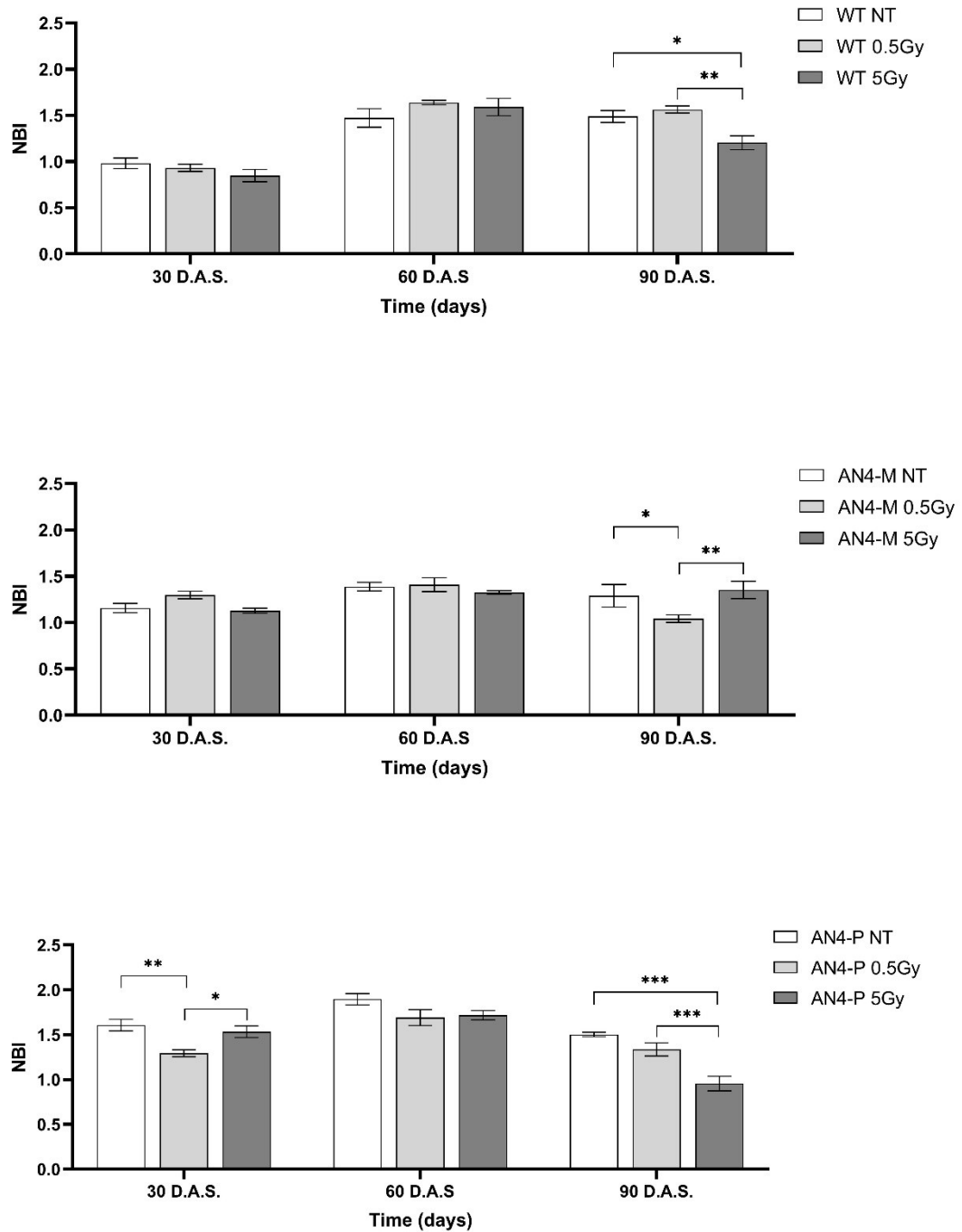


Figure 4. 74 Analysis of the NBI index of wild type, AN4-M and AN4-P₂ MicroTom along the observation period conducted on the offspring obtained from plants irradiated at 30 DAS. Statistical relevance: *: $p < 0.05$; **: $p < 0.001$; ***: $p < 0.0001$.

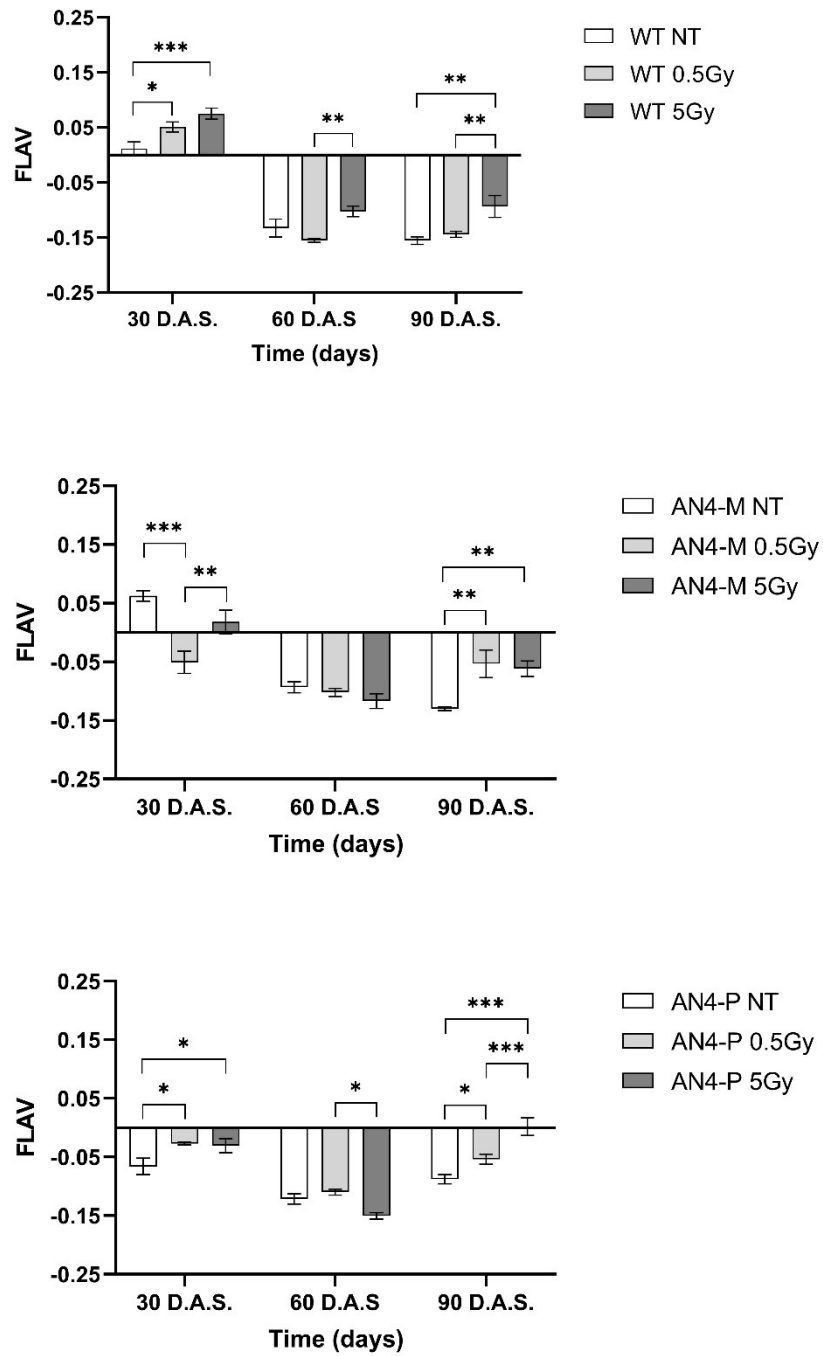


Figure 4. 75 Analysis of the FLAV index of wild type, AN4-M and AN4-P₂ MicroTom along the observation period conducted on the offspring obtained from plants irradiated at 30 DAS. Statistical relevance: *: $p < 0.05$; **: $p < 0.001$; ***: $p < 0.0001$.

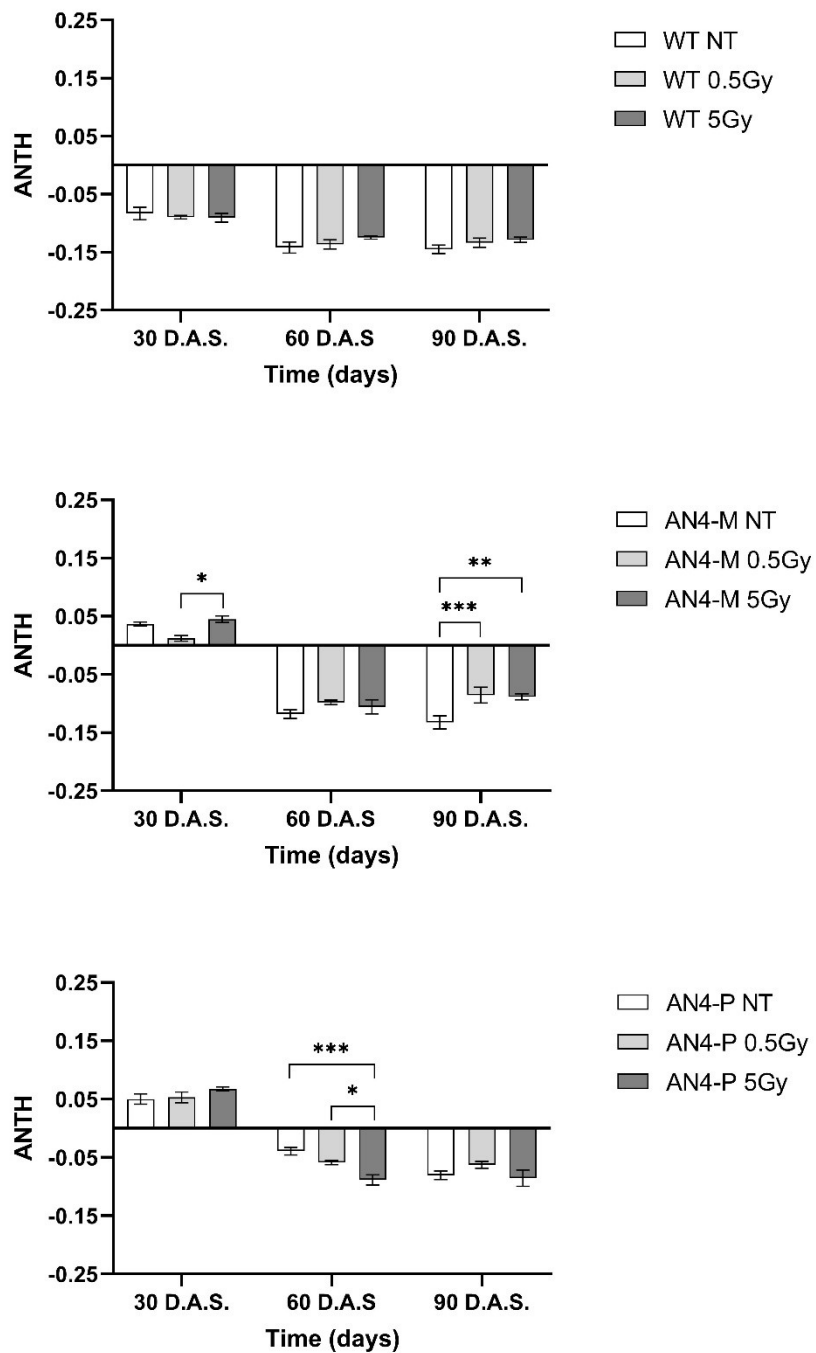


Figure 4. 76 Analysis of the ANTH index of wild type, AN4-M and AN4-P₂ MicroTom along the observation period conducted on the offspring obtained from plants irradiated at 30 DAS. Statistical relevance: *: $p < 0.05$; **: $p < 0.001$; ***: $p < 0.0001$.

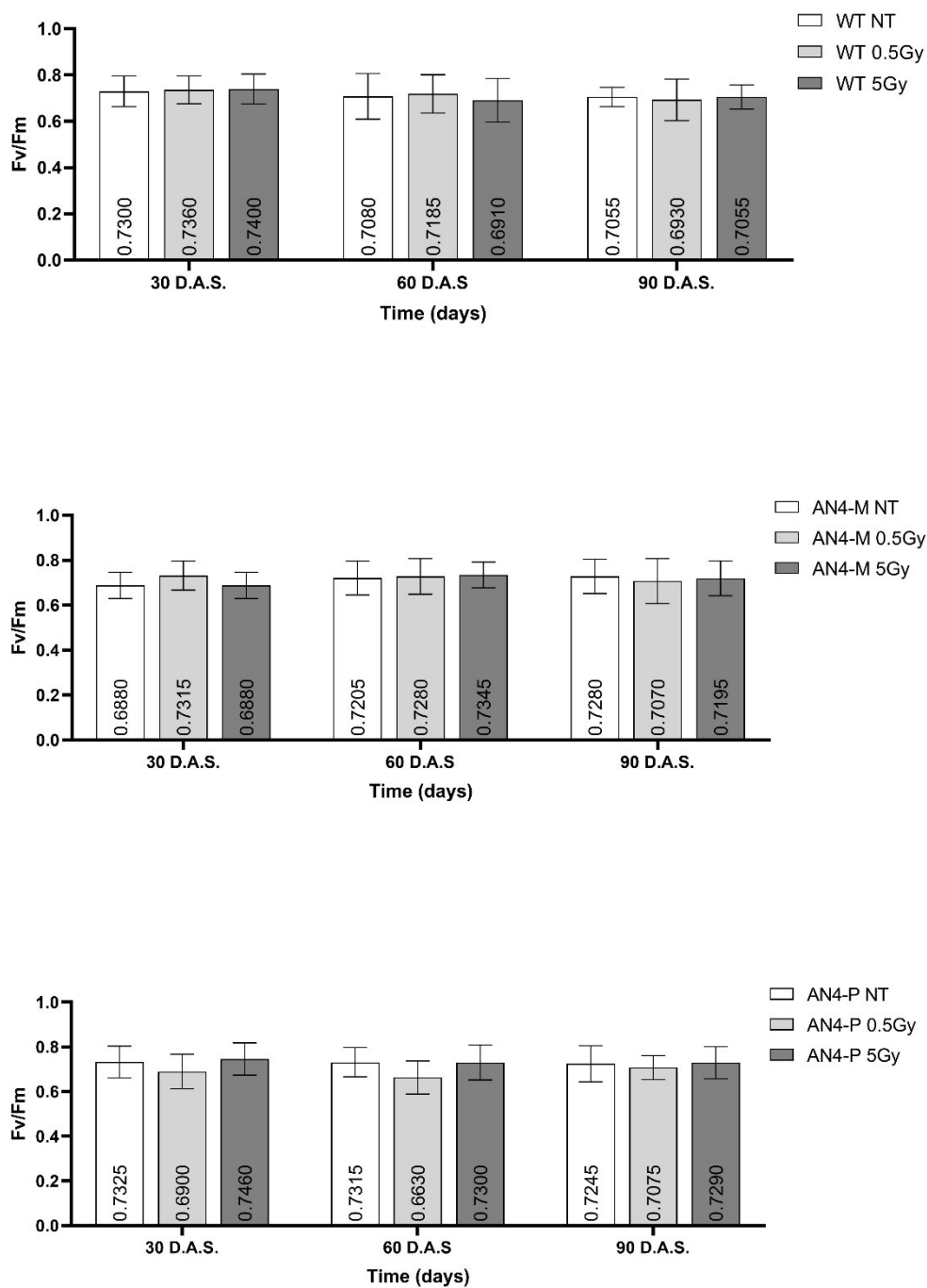


Figure 4. 77 Analysis of the Fv / Fm index of wild type, AN4-M and AN4-P₂ MicroTom along the observation period conducted on the offspring obtained from plants irradiated at 30 DAS. Statistical relevance: *: $p < 0.05$; **: $p < 0.001$; ***: $p < 0.0001$.

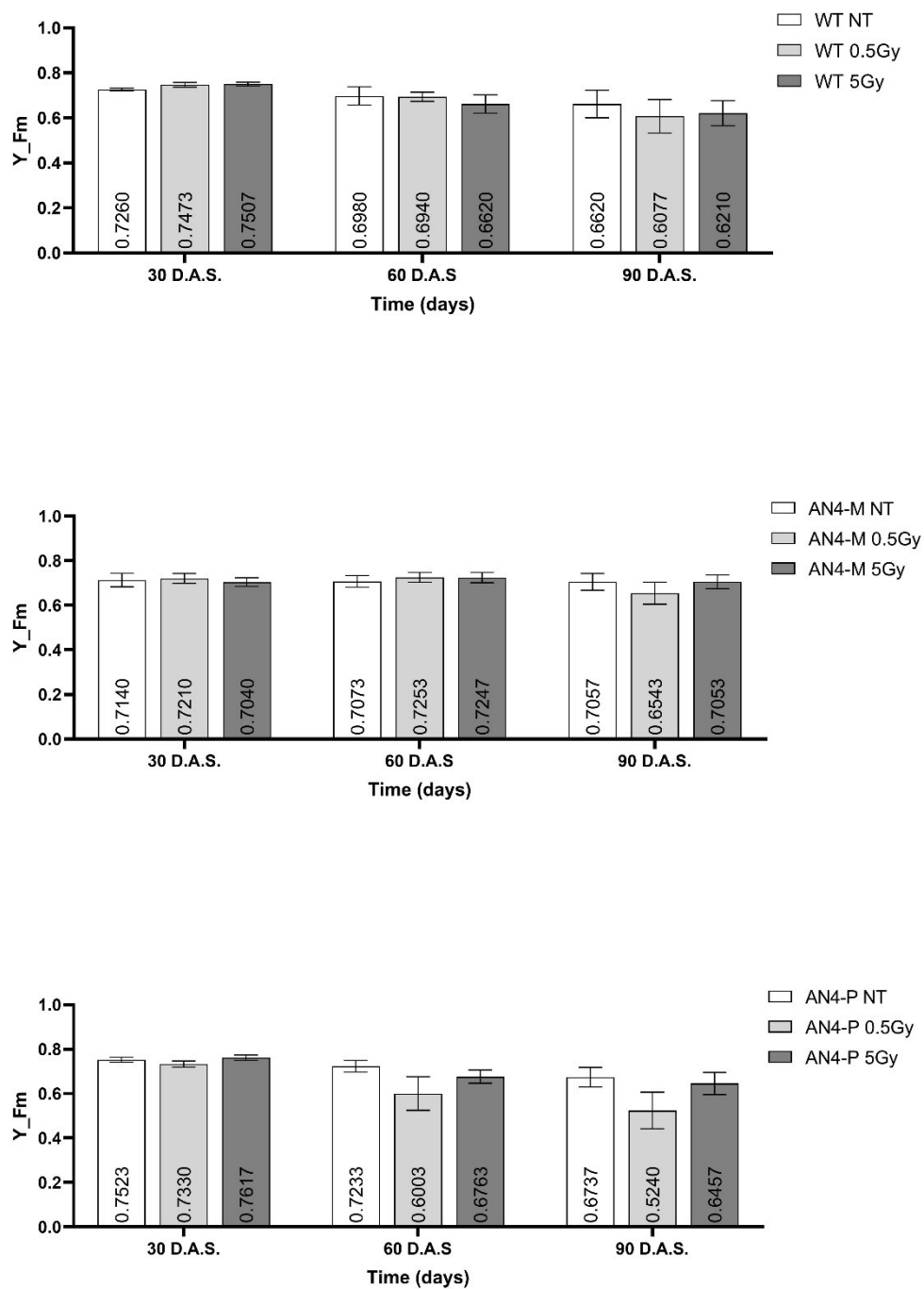


Figure 4. 78 Analysis of the Y_Fm index of wild type, AN4-M and AN4-P₂ MicroTom along the observation period conducted on the offspring obtained from plants irradiated at 30 DAS. Statistical relevance: *: $p < 0.05$; **: $p < 0.001$; ***: $p < 0.0001$.

4.6.3 Biometric and fluorimetric characterization of plants irradiated at 45 DAS

The 45 days after sowing (45 DAS) life stage represents the specific period at which MicroTom has completed approximately 50% of its entire life cycle. At this life stage MicroTom is almost ready to initiate the flowering time, that is pivotal for subsequent fruit set. PCA analysis was performed on plants subjected to gamma irradiation at 45 DAS (Figure 4.79). Due to the presence of missing values (i.e., low number of flowers and fruits) in the 30 Gy data matrix, the PCA analysis was performed on dataset obtained from plants irradiated with the two low doses (0.5 and 5 Gy).

The contributions of the first two PCs were 33.44% (PC1) and 17.94% (PC2), respectively, and their sum explained the 51.38% of the phenotypic variation. The loadings plot (Figure 4.79A) shows that FLAV, ANTH, number of fruits and number of flowers are positively correlated with the PC1. Leaf area, diameter of fruits, number of seeds but also number of flowers are positively correlated with the PC2. Conversely, the number of leaves, SFR and NBI resulted negatively correlated with both PC1 and PC2.

The score plot and the biplot (Figure 4.79B and Figure 4.79C) showed differential clustering among outcomes from the three genotypes as in the previous experiments. Wild type plants (both irradiated and not-irradiated samples) clustered in the lower-left quadrant (negative correlation of both PC1 and PC2). AN4-M plants were found close to the positive values of the y axis, showing their limited contribution to the variation of PC1 and main contribution to positive variations of the PC2. Finally, AN4-P₂ plants were found in the lower right corner positively correlating with PC1 and negatively with PC2.

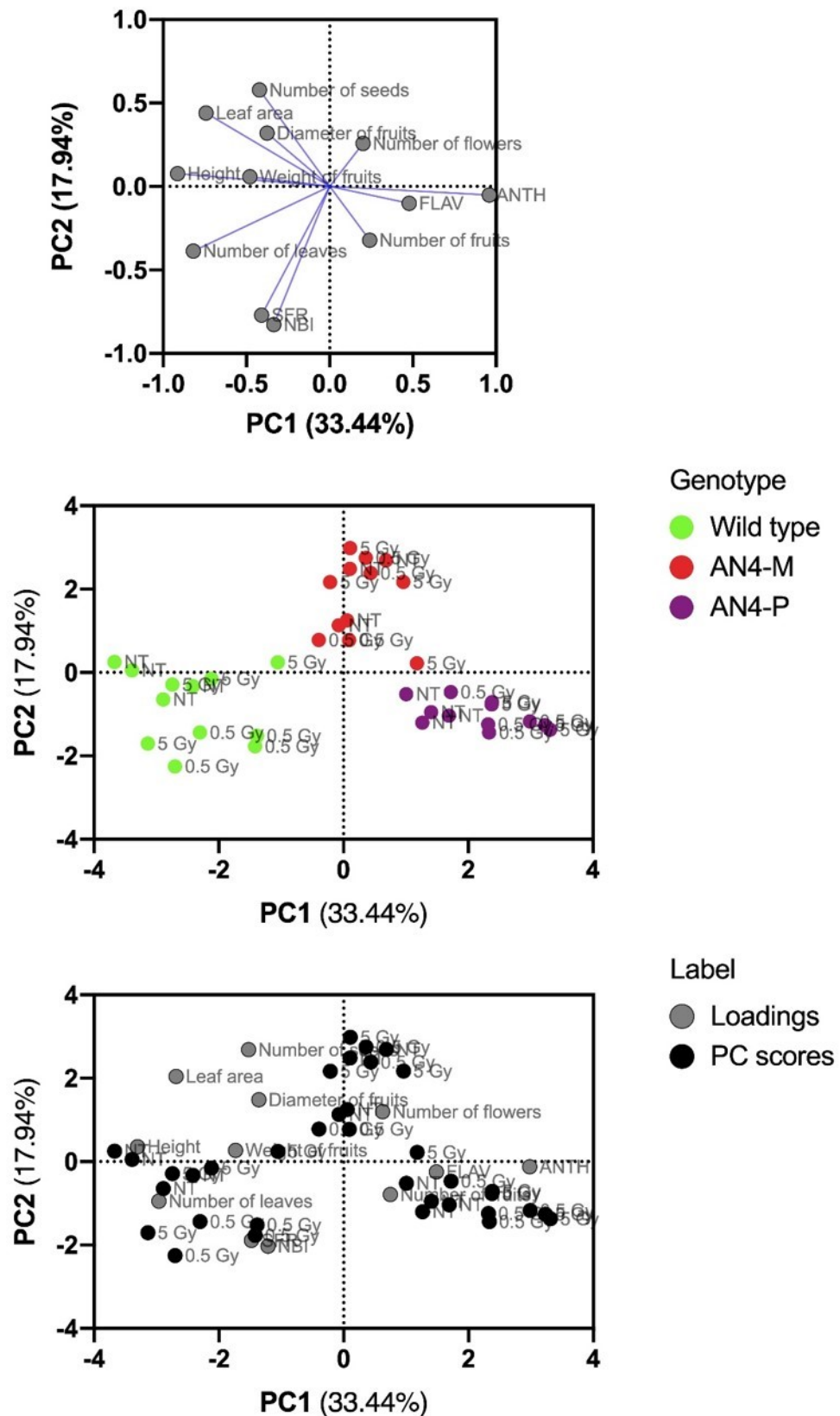


Figure 4. 79 Principal component analysis on morphometric and fluorimetric parameters obtained from plants irradiated at 45 DAS. The sum of PC1 (33.44%) and PC2 (17.94%) explained the 51.38% of the phenotypic variation. Results are graphically reported using the Loadings plot with all the parameters considered (A), the PC scores plot with wild type and AN4 plants (B) and the biplot that contains the information presented in both the Loadings plot and the PC scores plot at the same time (C).

From the analysis of variance (two-way Anova), and as expected, the acute irradiation at 30 Gy determined the overall most evident negative effects on both vegetative and yield traits independently of genotype (Figure 4.80). The non-significant interaction between ‘time’ and ‘gamma irradiation’ variables did not exceed the 14.4% of the total variation. The % of total variation ascribed to the ‘gamma irradiation’ variable resulted included between 1.96% and 18.5%, similar to that obtained for plants irradiated at 30 DAS and their offspring but higher in relation to that found for irradiated seeds. Finally, the % of variation ascribed to the ‘time’ variable was included between 50.8% and 92.4%, similar to that found for the other experiments.

	WT NT	WT 0.5Gy	WT 5Gy	WT 30Gy	
Height (cm)	14.67±0.7	14.22±0.25	14.23±0.43	12.43±0.81	+ ***
Number of leaves	40.4±2.01	41±1.14	46.8±0.58	30.8±1.59	+ **
Leaf area (mm ²)	210.68±1.08	197.01±5.01	205.69±4.07	147.08±15.69	+ *
Number of flowers	18.8±1.15	19.6±2.37	20.2±2.33	2.2±0.48	/
Number of fruits	17.6±0.67	19.8±0.96	23±0.89	1±1	- *
Weight of fruits (g)	3.08±0.24	2.53±0.22	2.78±0.18	0.34±0.18	- **
Diameter of fruits (mm)	18.37±0.74	16.28±0.79	19.9±1.61	3.34±1.7	- ***
Number of seeds	20±0.53	12.66±0.45	22.53±0.35	/	

	AN4-M NT	AN4-M 0.5Gy	AN4-M 5Gy	AN4-M 30Gy
Height (cm)	11.89±0.67	11.67±0.35	11.74±0.46	10.65±0.18
Number of leaves	18±0.54	19.2±1.28	13.6±1.12	15.4±0.67
Leaf area (mm ²)	203.75±10.3	191.55±7.64	194.72±18.53	120.59±20.89
Number of flowers	21.6±0.67	21.2±1.31	20.6±1.2	2.6±0.24
Number of fruits	19.8±0.58	19.8±0.91	19.4±1.28	1±1
Weight of fruits (g)	2.47±0.17	2.02±0.21	2.68±0.19	0.26±0.18
Diameter of fruits (mm)	17.84±0.5	17.41±0.69	16.77±0.77	4.33±2.24
Number of seeds	28.8±1.3	20.46±0.56	21.46±1.09	/

	AN4-P NT	AN4-P 0.5Gy	AN4-P 5Gy	AN4-P 30Gy
Height (cm)	8.69±0.2	9.17±0.22	8.37±0.27	7.82±0.74
Number of leaves	18.8±1.98	21.2±1.39	16.4±1.72	14±2.54
Leaf area (mm ²)	123.66±7.26	131.93±4.5	114.34±9.33	95.62±16.91
Number of flowers	20.8±0.58	20.4±0.92	20±1.51	4±0.31
Number of fruits	21.4±0.5	21.4±0.67	21±0.7	0.8±0.8
Weight of fruits (g)	2.24±0.23	1.75±0.18	1.4±0.2	0.48±0.21
Diameter of fruits (mm)	14.82±1.98	13.73±0.55	13.39±1.11	5.73±2.41
Number of seeds	18.66±0.47	11.13±0.84	6±0.33	/

Figure 4. 80 Summary of morphometric results at the end of the measurements period analysed in comparison within each genotypes and according to the different treatments on plants irradiated at 45 DAS. Data were subjected to two-way analysis of variance (two-way ANOVA) with Tukey’s post-hoc test to determine the differences in average of all tested parameters between irradiated and non-irradiated plants. NT: non-treated.

While the irradiation with 30 Gy dose produced negative effects on all the three genotypes (from Figure 4.81 to Figure 4.88), with the only exception of plant height in AN4-M and AN4-P₂ plants and leaf area in AN4-M plants, the 0.5 Gy dose negatively affected the number of seeds in all the three genotypes. The 5 Gy dose positively affected the number of leaves, number of fruits and number of seeds in wild type plants, while it negatively influenced the number of leaves of AN4-M plants and the weight of fruits of AN4-P₂ plants. To be noted that wild type and AN4-M plants showed statistically significant changes starting from 14 to 21 DAI (59 to 66 DAS) for plant height, number of leaves, leaf area and number of fruits, while AN4-P₂ plants revealed relevant changes only for number of flowers starting from 14 DAI (59 DAS). The major effects found during the measurement period were caused by the 30 Gy dose, as demonstrated by the data obtained at the end of the detection period.

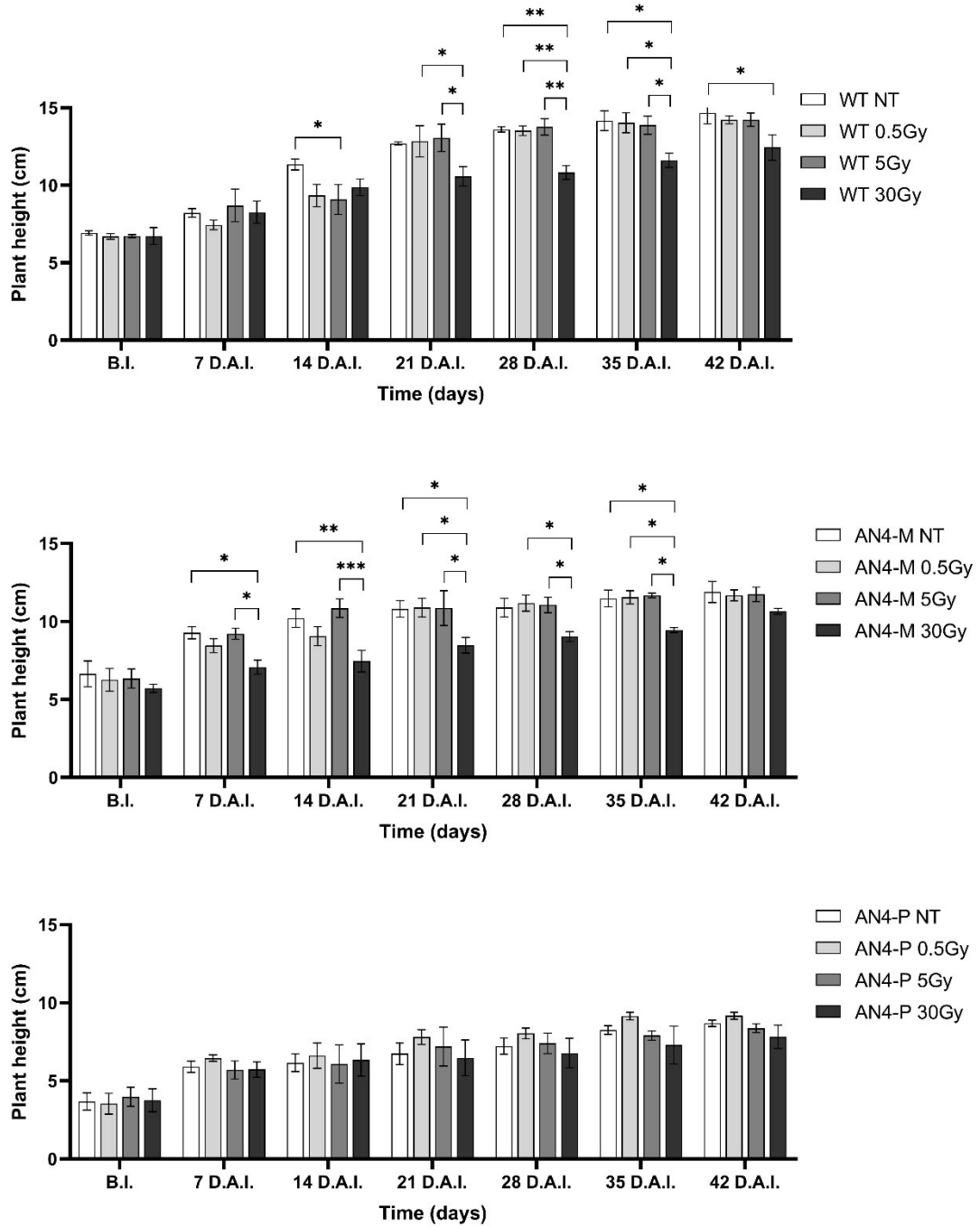


Figure 4. 81 Analysis of plant height of wild type, AN4-M and AN4-P₂ MicroTom along the observation period upon different irradiation treatments supplied to plants at 45 DAS. Statistical relevance: *: $p < 0.05$; **: $p < 0.001$; ***: $p < 0.0001$.

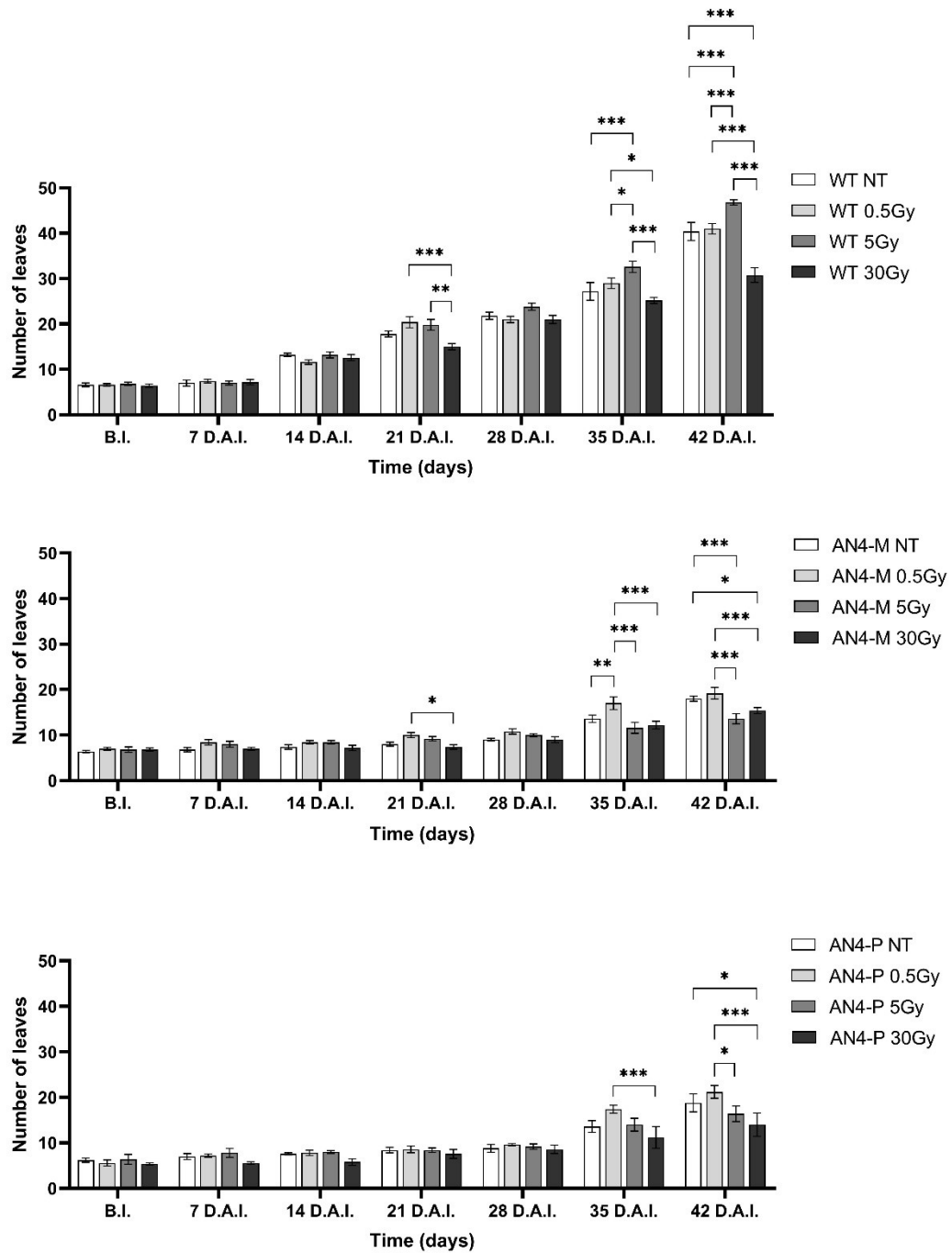


Figure 4. 82 Analysis of number of leaves of wild type, AN4-M and AN4-P₂ MicroTom along the observation period upon different irradiation treatments supplied to plants at 45 DAS. Statistical relevance: *: $p < 0.05$; **: $p < 0.001$; ***: $p < 0.0001$.

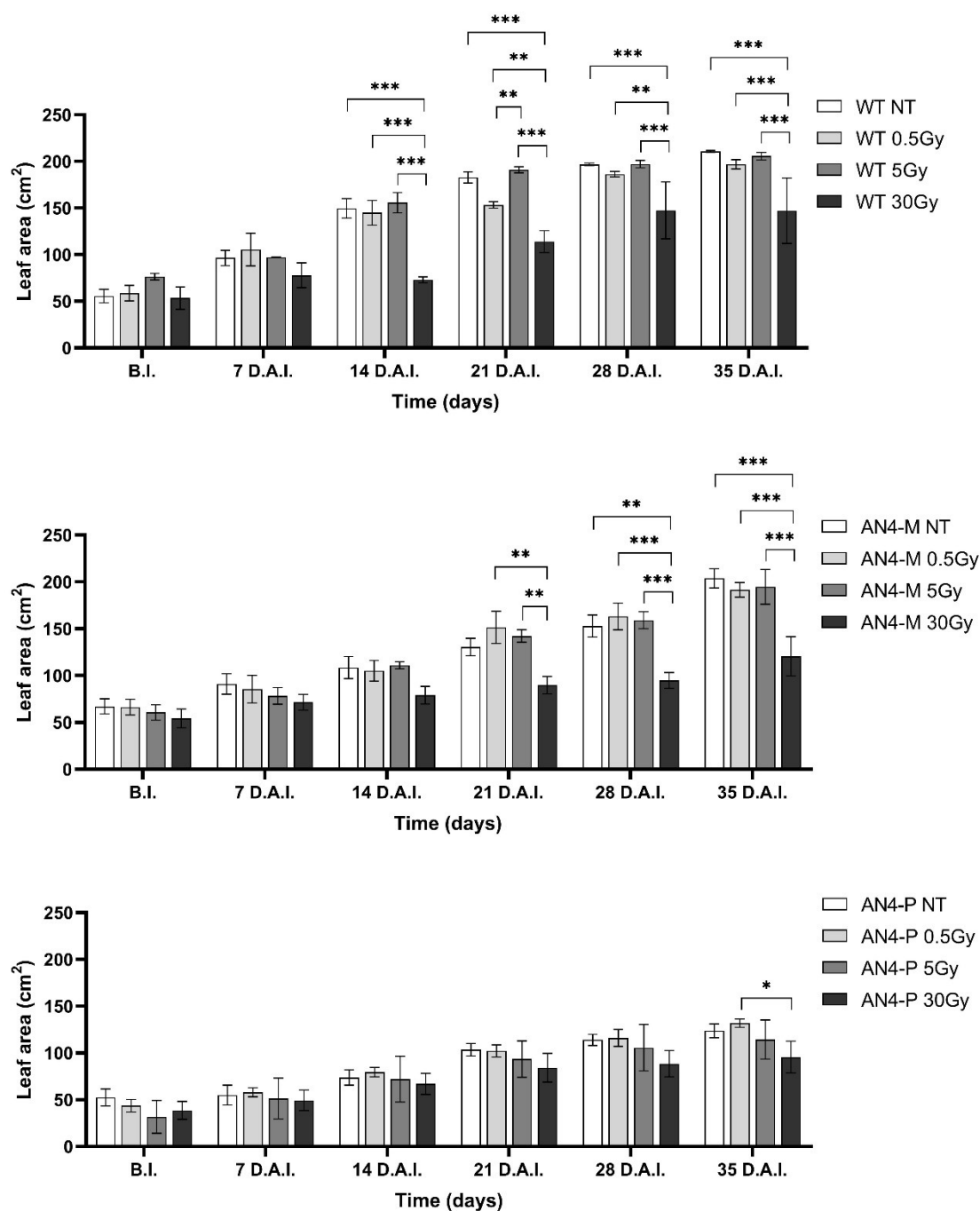


Figure 4. 83 Analysis of leaf area of wild type, AN4-M and AN4-P₂ MicroTom along the observation period upon different irradiation treatments supplied to plants at 45 DAS. Statistical relevance: *: $p < 0.05$; **: $p < 0.001$; ***: $p < 0.0001$.

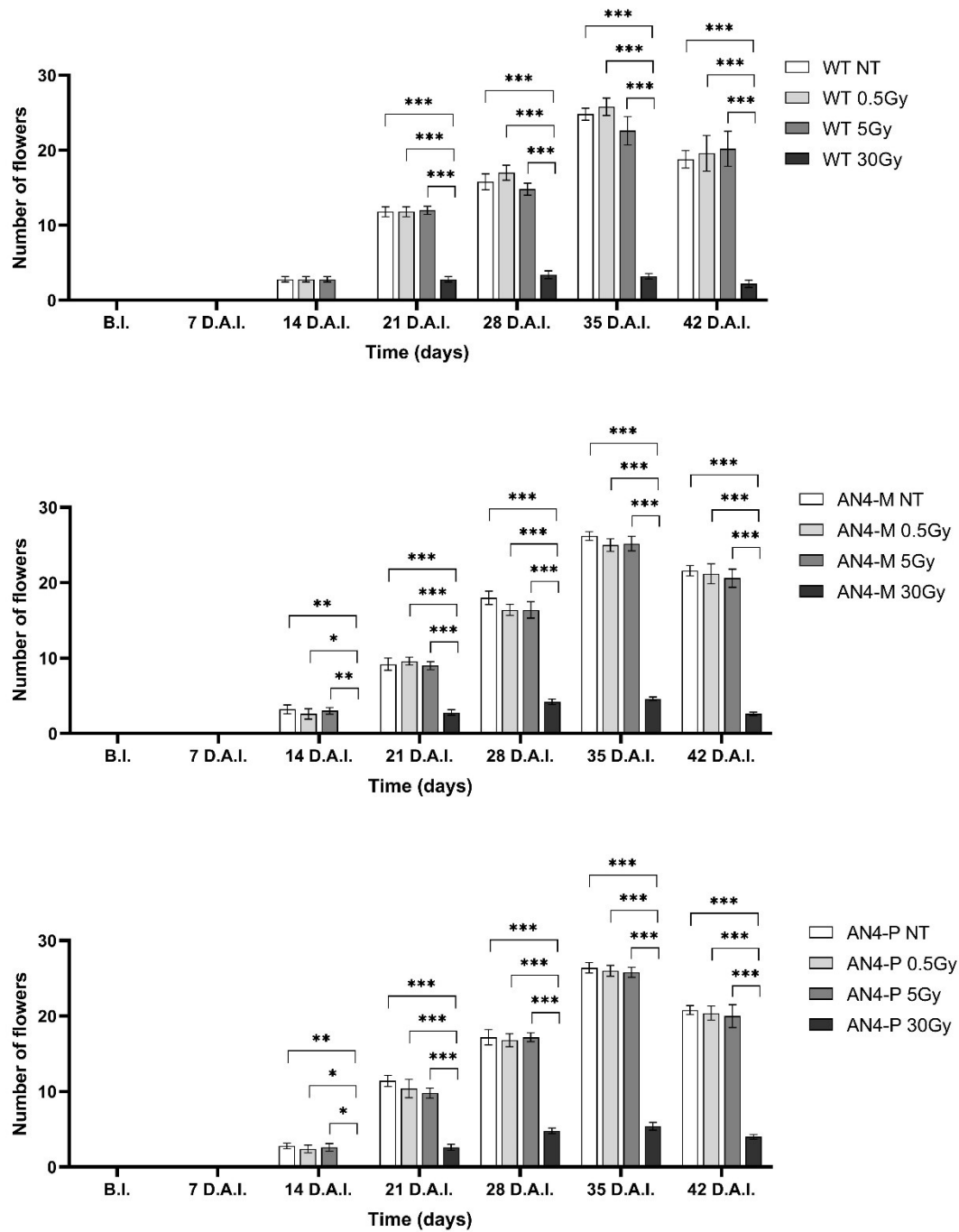


Figure 4. 84 Analysis of number of flowers of wild type, AN4-M and AN4-P₂ MicroTom along the observation period upon different irradiation treatments supplied to plants at 45 DAS. Statistical relevance: *: $p < 0.05$; **: $p < 0.001$; ***: $p < 0.0001$.

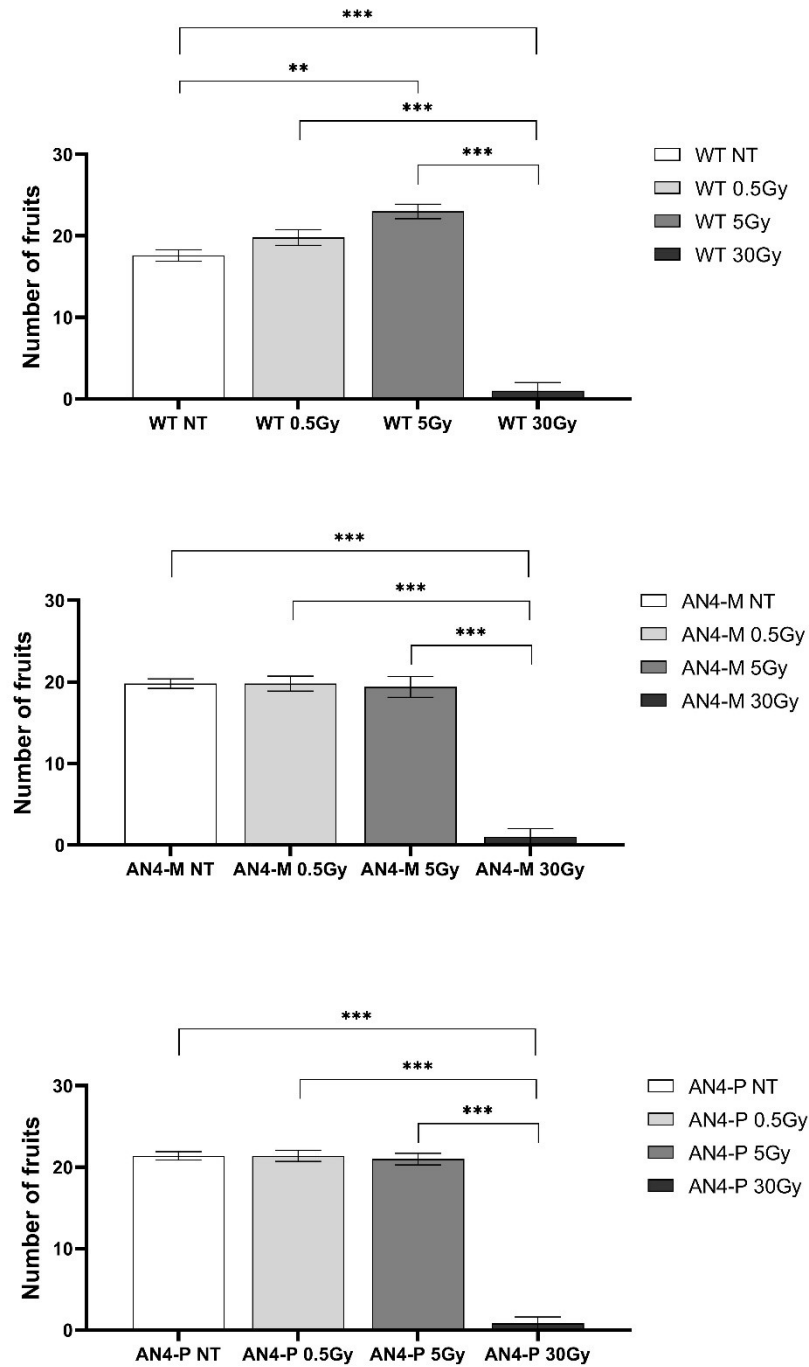


Figure 4. 85 Analysis of number of fruits of wild type, AN4-M and AN4-P₂ MicroTom along the observation period upon different irradiation treatments supplied to plants at 45 DAS. Statistical relevance: *: $p < 0.05$; **: $p < 0.001$; ***: $p < 0.0001$.

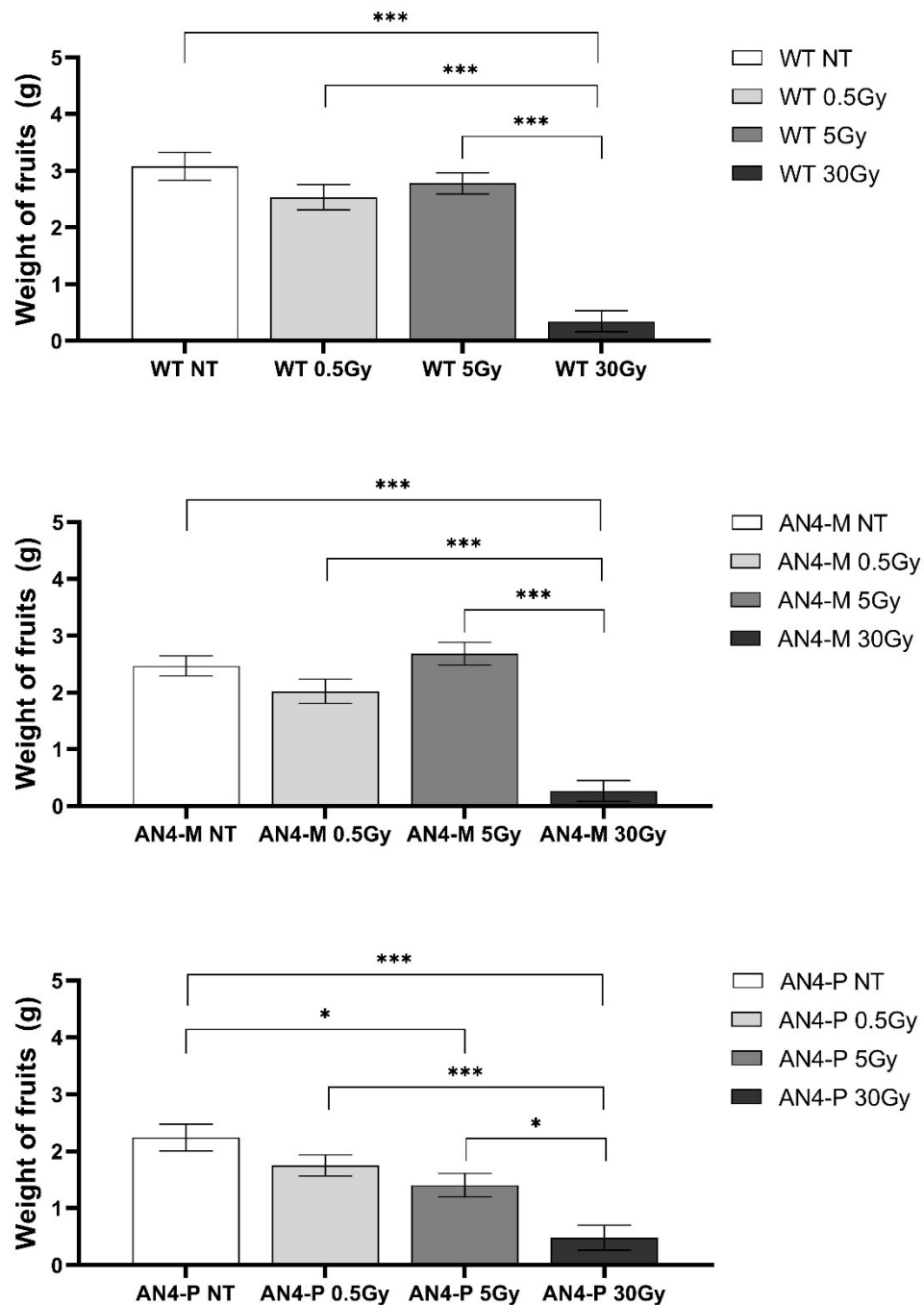


Figure 4. 86 Analysis of weight of fruits of wild type, AN4-M and AN4-P₂ MicroTom along the observation period upon different irradiation treatments supplied to plants at 45 DAS. Statistical relevance: *: $p < 0.05$; **: $p < 0.001$; ***: $p < 0.0001$.

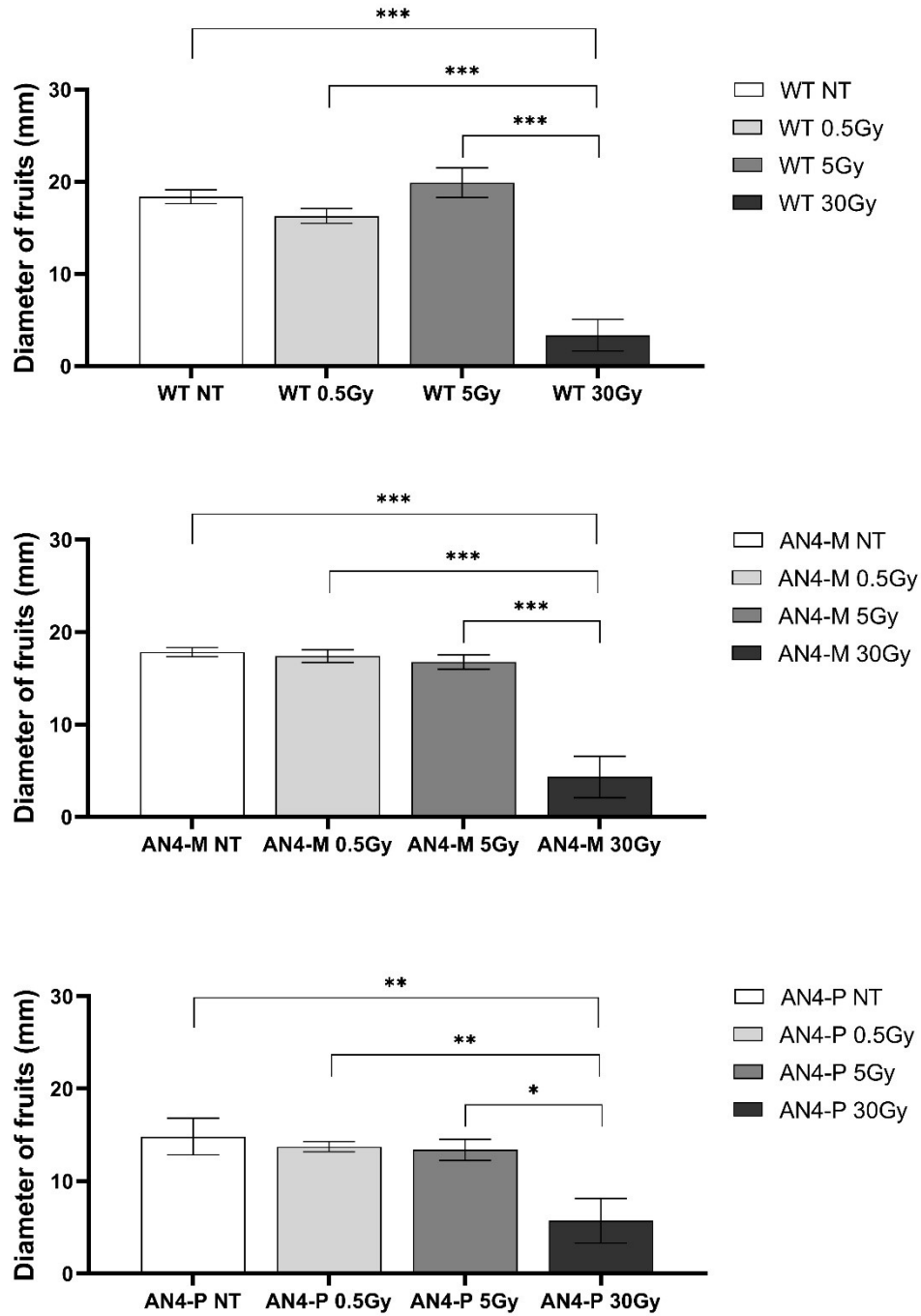


Figure 4. 87 Analysis of diameter of fruits of wild type, AN4-M and AN4-P₂ MicroTom along the observation period upon different irradiation treatments supplied to plants at 45 DAS. Statistical relevance: *: $p < 0.05$; **: $p < 0.001$; ***: $p < 0.0001$.

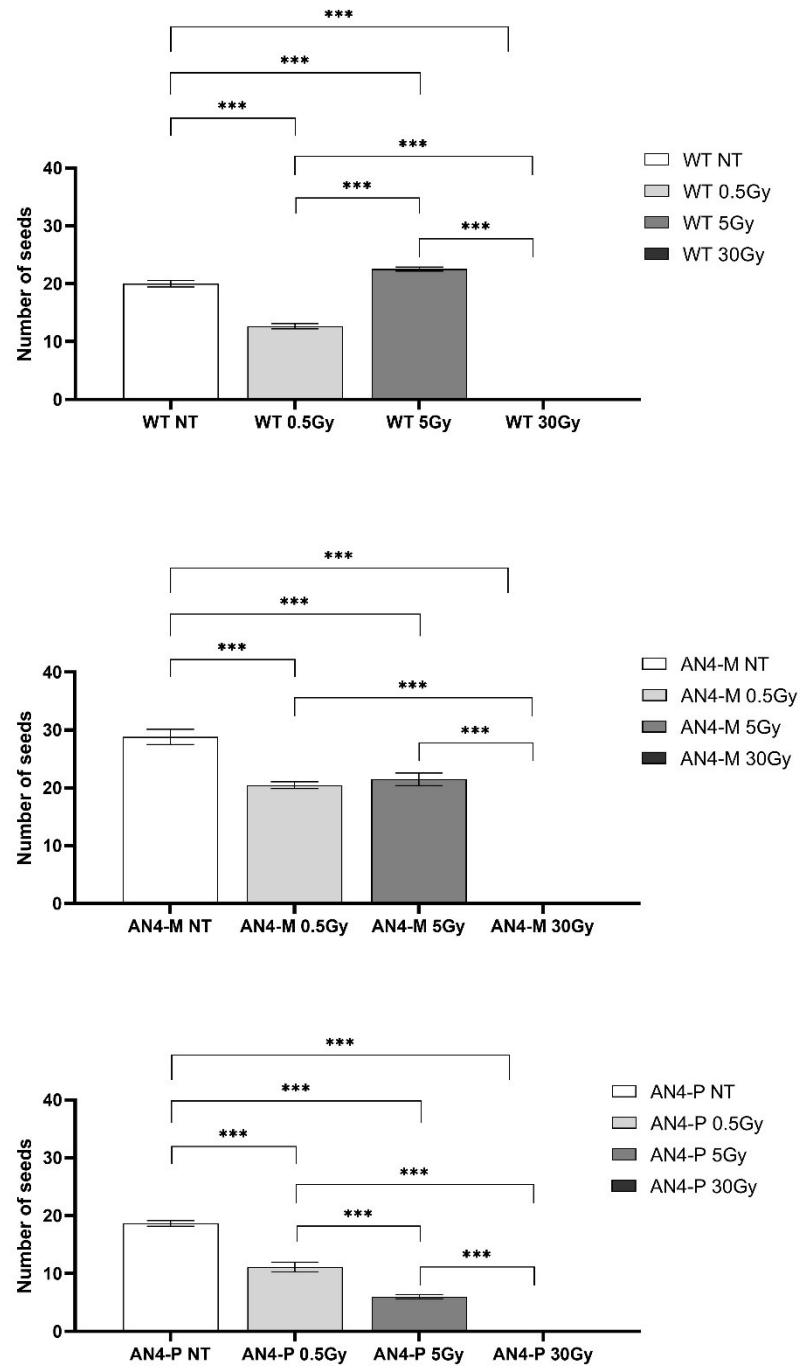


Figure 4. 88 Analysis of number of seeds of wild type, AN4-M and AN4-P₂ MicroTom along the observation period upon different irradiation treatments supplied to plants at 45 DAS. Statistical relevance: *: $p < 0.05$; **: $p < 0.001$; ***: $p < 0.0001$.

The values of fruits-related parameters at fruit set, were compared among the three genotypes in function of the radiation dose, as well (Figure 4.89) and, in accordance with data obtained from from irradiated seeds and plants irradiated at 30 DAS, no statistical variation was observed in number of fruits. Conversely, 0.5 and 5 Gy doses negatively affected the weight and diameter of fruits of AN4-P₂ plants. On the contrary, the three genotypes are significantly different for number of seeds already in standard conditions. Irradiation with 0.5 Gy caused a decrease in number of seeds for all the three genotypes while the 5 Gy dose had this negative effect only in AN4 plants.

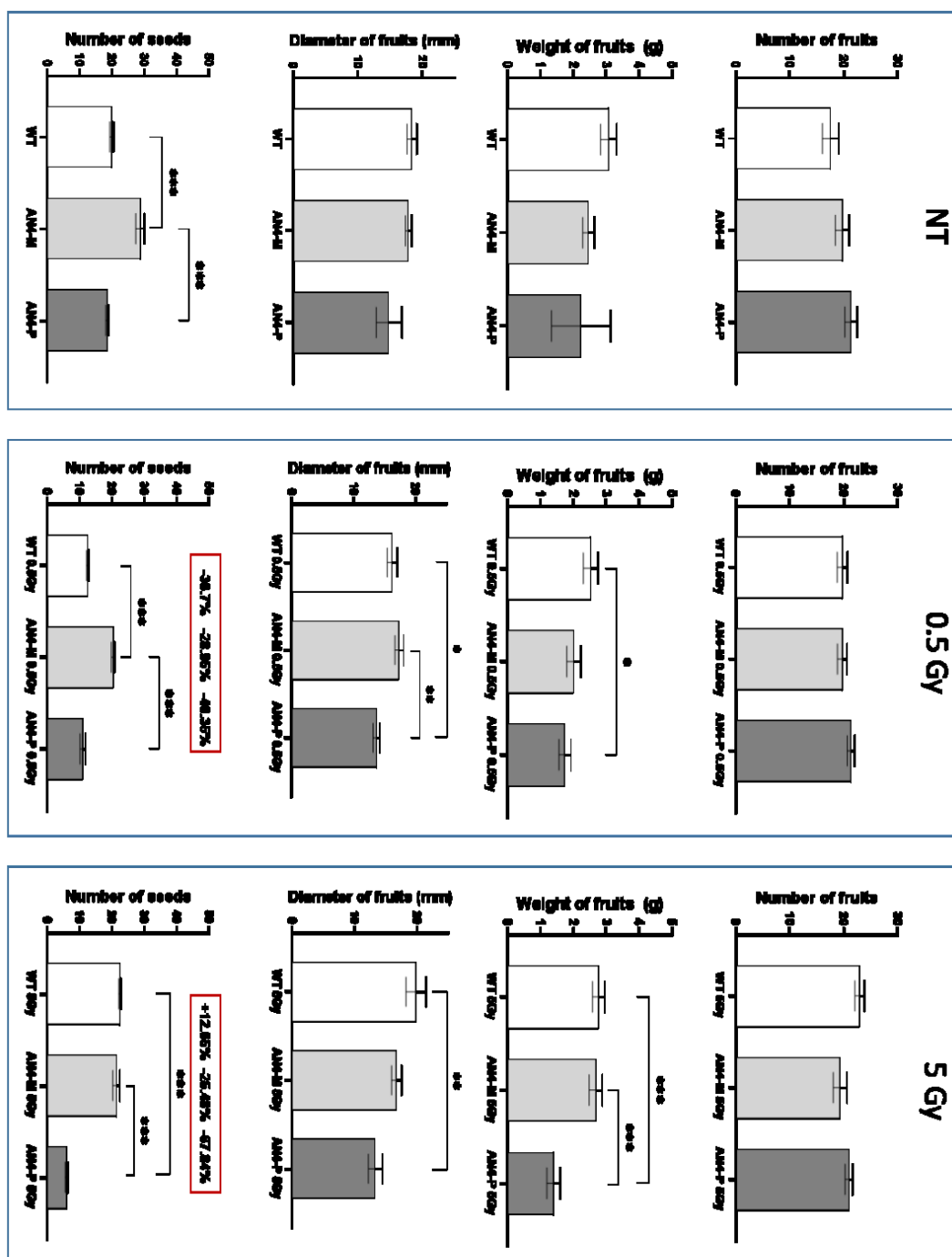


Figure 4. 89 Comparison of fruits-related parameters among the three genotypes according to the different treatments on plants irradiated at 45 DAS.

In relation to the fluorimetric parameters measured with the Multiplex Force A™ (SFR, FLAV, ANTH, and NBI), the irradiation of plants at 45 DAS produced some significant effects (Figure 4.90). The % of total variation due to the ‘gamma irradiation’ variable spaced between 2.6% and 27.3%, similar to that found for plants irradiated at 30 DAS and higher than that found in irradiated seeds, while the ‘time’ variable resulted between 15.1% and 84.2%. The not significant interaction between the two variables was always between 2.6% and 27.3%. At the end of the detection period, only positive variations of the FLAV index were recorded. This happened in wild type plants, independently of the dose supplied, and in AN4-P₂ plants supplied with the 0.5 Gy dose. No variation of indices was recorded in AN4-M plants.

	WT NT	WT 0.5Gy	WT 5Gy	WT 30Gy	
SFR	2.14±0.04	2.27±0.06	1.99±0.04	2.09±0.03	+ ***
FLAV	-0.15±0.01	-0.13±0.001	-0.14±0.007	-0.05±0.01	+ **
ANTH	-0.15±0.007	-0.13±0.001	-0.14±0.01	-0.14±0.01	+ *
NBI	2.03±0.02	2.17±0.06	2.09±0.07	2.03±0.1	/
					- *
					- **
					- ***
	AN4-M NT	AN4-M 0.5Gy	AN4-M 5Gy	AN4-M 30Gy	
SFR	1.93±0.04	1.8±0.06	1.88±0.07	2±0.01	
FLAV	-0.1±0.003	-0.13±0.006	-0.1±0.01	-0.11±0.01	
ANTH	-0.1±0.001	-0.11±0.001	-0.09±0.004	-0.09±0.005	
NBI	1.83±0.06	1.86±0.05	1.86±0.07	1.99±0.03	
	AN4-P NT	AN4-P 0.5Gy	AN4-P 5Gy	AN4-P 30Gy	
SFR	2.02±0.03	2.02±0.03	1.85±0.02	2.05±0.01	
FLAV	-0.13±0.006	-0.08±0.006	-0.13±0.005	-0.08±0.01	
ANTH	-0.09±0.004	-0.06±0.001	-0.07±0.004	-0.06±0.01	
NBI	2.07±0.07	1.94±0.007	1.98±0.01	2.01±0.01	

Figure 4. 90 Summary of fluorimetric results at the end of the measurements period analysed in comparison within each genotypes and according to the different treatments supplied to plants at 45 DAS. Data were subjected to two-way analysis of variance (two-way ANOVA) with Tukey’s post-hoc test to determine the differences in average of all tested parameters between irradiated and non-irradiated plants. NT: non-treated.

Throughout the whole measurement period, the values detected for the SFR and NBI indices (Figure 4.91 and Figure 4.92) showed a periodic sigmoidal-like trend progressively increasing until the end of measurements independently of genotypes (even if with different inflection

points) and treatment. Wild type plants showed negative effects on SFR at 35 DAI (80 DAS) and negative effects at 42 DAI (87 DAS), while AN4-M plants showed mostly negative effects from 3h AI to 24h AI, and positive effects from 21 DAI (66 DAS) to 42 DAI (94 DAS). In particular, the 30 Gy dose positively influenced the SFR index only at 21 DAI (66 DAS) while the 5 Gy dose had a positive effect from 21 to 42 DAI (66-94 DAS). Moreover, the 0.5 and 5 Gy doses positively influenced the SFR index of AN4-P₂ plants only at 35 DAI (79 DAS).

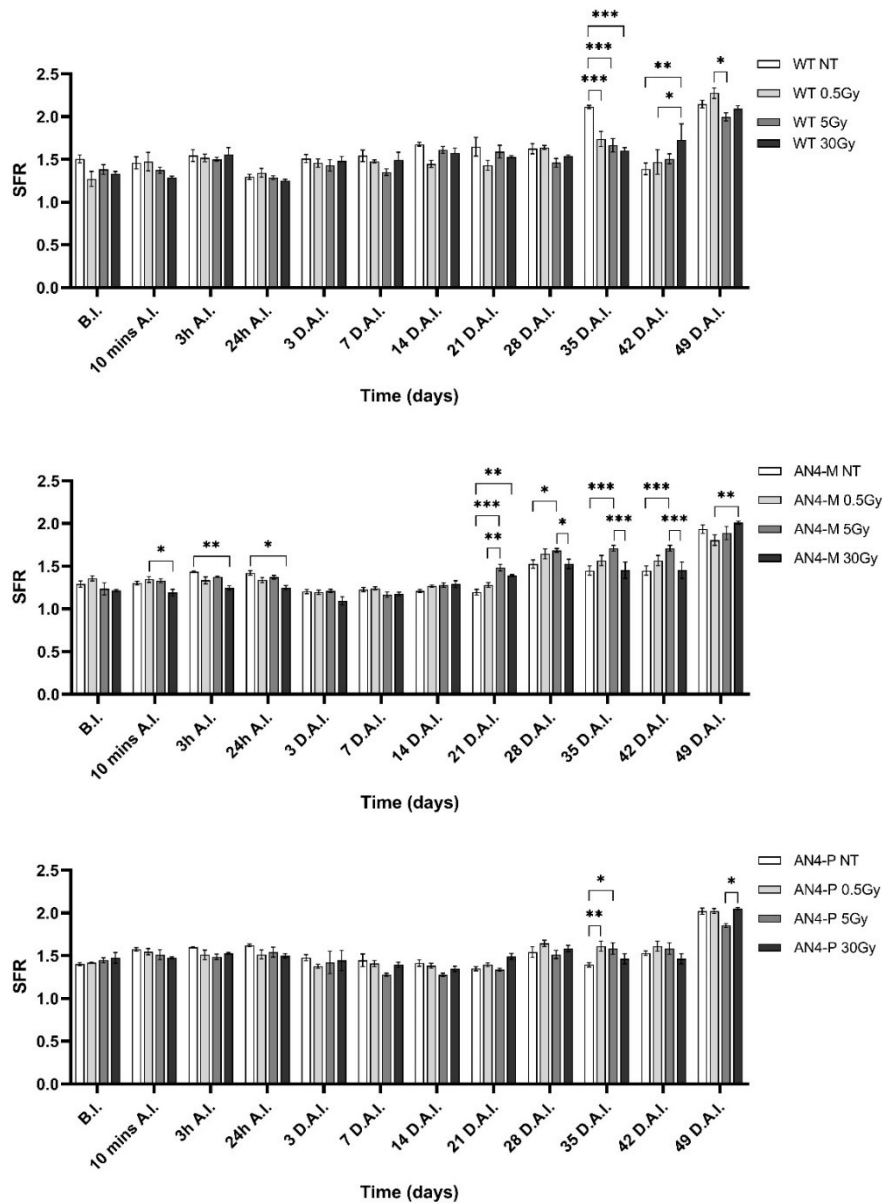


Figure 4.91 Analysis of the SFR index of wild type, AN4-M and AN4-P₂ MicroTom along the observation period upon different irradiation treatments supplied to plants at 45 DAS. Statistical relevance: *: $p < 0.05$; **: $p < 0.001$; ***: $p < 0.0001$.

While NBI did not reveal significant variations for the wild type plants compared to non-treated controls and independently from the dose, significant positive differences were recorded for

AN4-M irradiated with 0.5 and 5 Gy doses at 21 DAI (66 DAS). Conversely, negative effects were found for both AN4-M and AN4-P₂ genotypes irradiated with 30 Gy starting from 28 and 35 DAI, respectively.

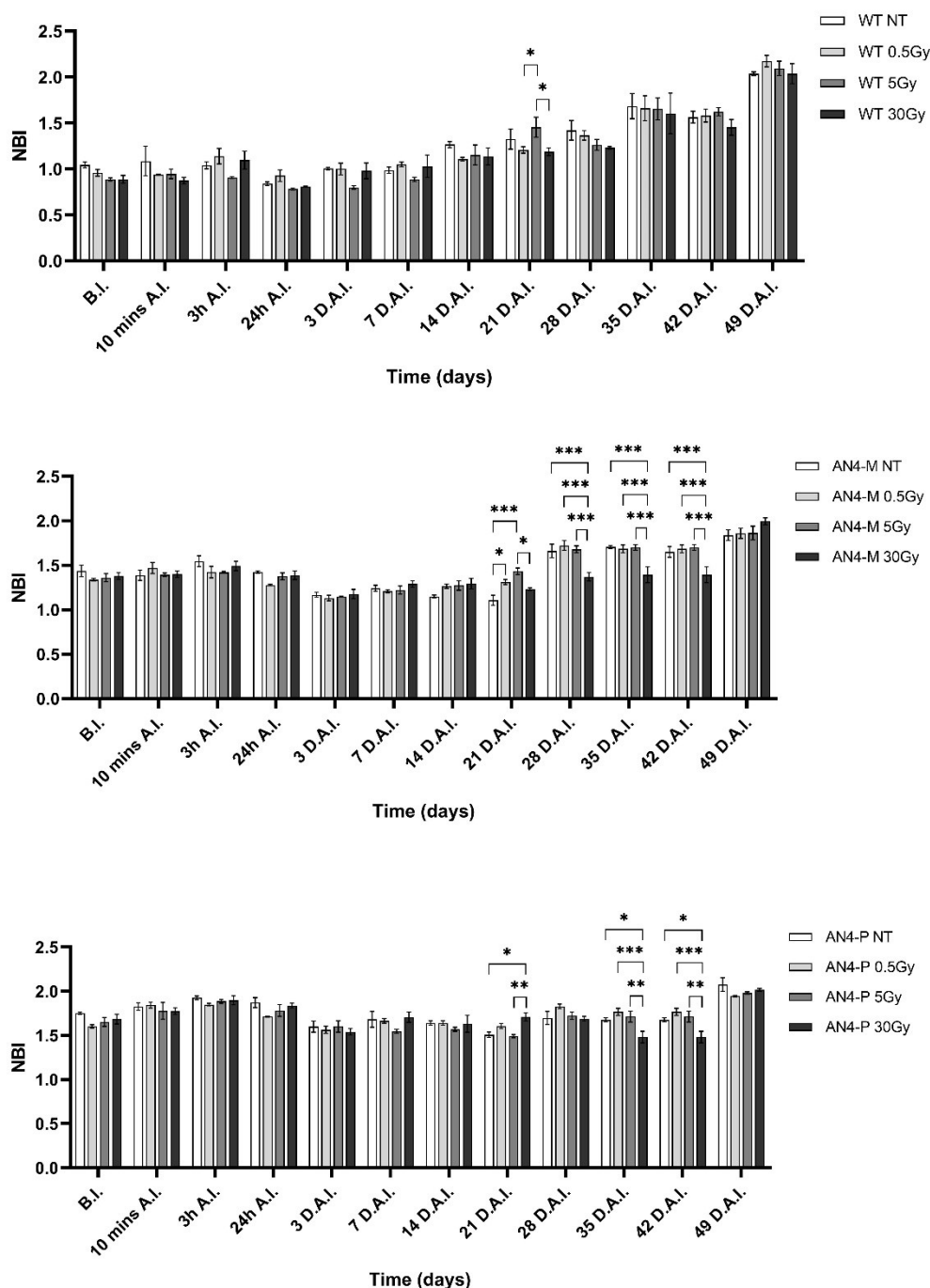


Figure 4.92 Analysis of the NBI index of wild type, AN4-M and AN4-P₂ MicroTom along the observation period upon different irradiation treatments supplied at 45 DAS. Statistical relevance: *: $p < 0.05$; **: $p < 0.001$; ***: $p < 0.0001$.

The FLAV index values recorded throughout the measurement period showed a sigmoidal-like shape trend in wild type with a value inversion point between 7 and 14 DAI (52-59 DAS very different from that of 37-44 DAS seen in plants irradiated at 30 DAS plants) (Figure 4.93). On

the contrary, the FLAV index values were registered as constantly negative for AN4 plants, probably reflecting an anticipated decrease of flavonols levels as precursors for a boosted biosynthesis of anthocyanins. Interestingly, while several changes of the FLAV index were found in wild type plants from 35 to 49 DAI (80 to 94 DAS), these changes resulted as an early response in AN4-P₂ plants, especially when subjected to the 0.5 Gy dose. Moreover, at 42 DAI (87 DAS) the 30 Gy dose positively affected the FLAV index of AN4-P₂ plants. With the only exception of the 0.5 Gy dose effect found at 21 DAI (66 DAS), no changes occurred between treatments and control in AN4-M plants.

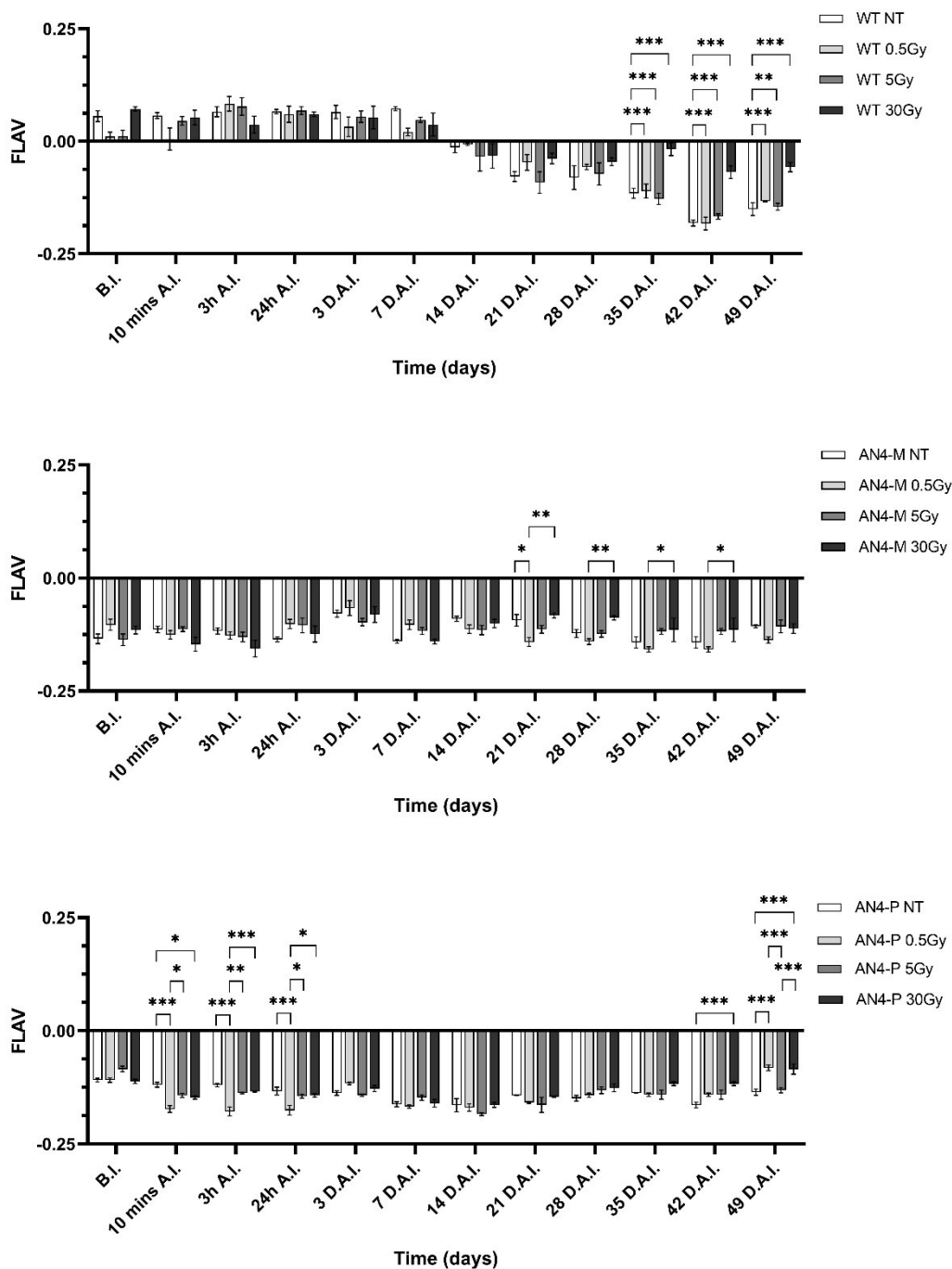


Figure 4. 93 Analysis of the FLAV index of wild type, AN4-M and AN4-P₂ MicroTom along the observation period upon different irradiation treatments supplied at 45 DAS. Statistical relevance: *: p<0.05; **: p<0.001; ***: p<0.0001.

The ANTH index values defined differential curves for the wild type compared to the two transgenic genotypes (Figure 4.94). Indeed, while ANTH values were constant and negative throughout life cycle in wild type descendants, indicating absence of anthocyanins, AN4 plants were characterized by a sigmoidal curve with a value inversion point BI (before 45 DAS, similar to that of 37 and 44 DAS of the 30 DAS plants), indication of flavonols dilution in the tissues in the second half of the life cycle in parallel with the increase of vegetative growth. Along the detection period, the 5 Gy dose resulted in a decrease of the ANTH index of wild type plants between 3h AI and 3 DAI (48 DAS). Conversely, an increase of the ANTH index was found at 3 DAI in AN4-P₂ plants irradiated with the 5 Gy dose. Moreover, the irradiation positively influenced the ANTH index in AN4-M plants, especially at 7 DAI (5 Gy dose), at 35 and 42 DAI (30 Gy dose).

In addition, along the detection period, it was possible to detect differences between responses of plants irradiated either at 30 DAS or at 45 DAS. While AN4-M plants responded with a clear increase in the FLAV index compared to untreated and the lower doses when irradiated at 30 DAS, the same plants did not respond at all when irradiated at 45 DAS. The same difference between 30 and 45 DAS was detectable for the ANTH values, as well. Even though less pronounced, the same ‘lack of response’ was noticed for SFR and NBI as if these were age-related responses to the irradiation stress.

Finally, the Fv/Fm and Y_Fm indices always resulted in the range described by the Plant Stress Kit (PSKII) manufacturer (0.7/0.9 for healthy leaves) but some statistically differences were noted. In particular, Fv/Fm resulted in a slight decrease for AN4-P₂ plants treated with the 30 Gy dose after 10 minutes from the irradiation, with an increase during the following 3 hours (Figure 4.95). Conversely, the Y_Fm showed several changes starting from 10 minutes after irradiation, although in the range of the healthy status, only for AN4 plants (Figure 4.96).

Summarizing, gamma rays supplied to plants at 45 DAS caused an increase in FLAV indices in wild type plants and minor changes in AN4-P₂ plants. No changes occurred in AN4-M plants, re-confirming the greater stability of this genotype.

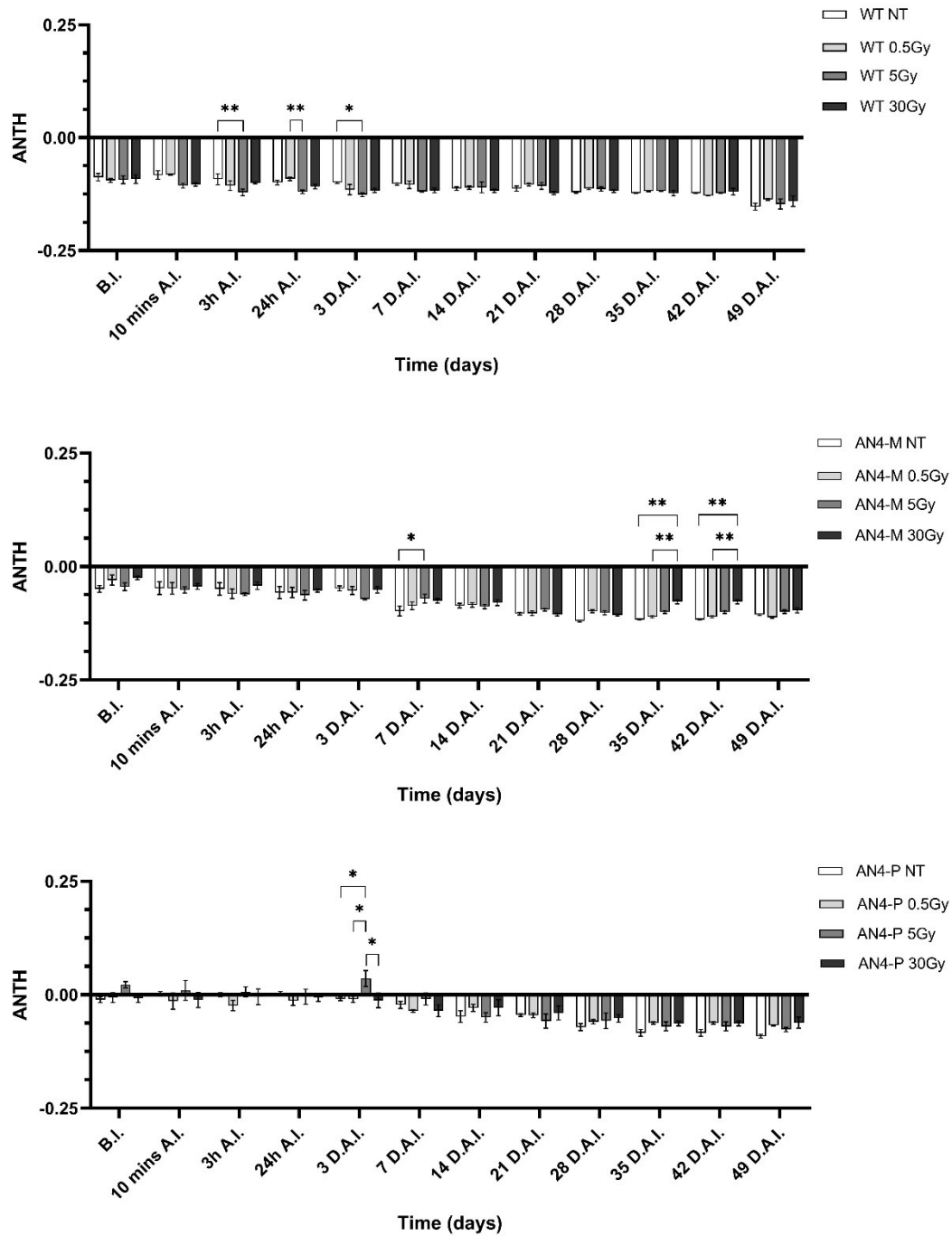


Figure 4. 94 Analysis of the ANTH index of wild type, AN4-M and AN4-P₂ MicroTom along the observation period upon different irradiation treatments supplied at 45 DAS. Statistical relevance: *: $p < 0.05$; **: $p < 0.001$; ***: $p < 0.0001$.

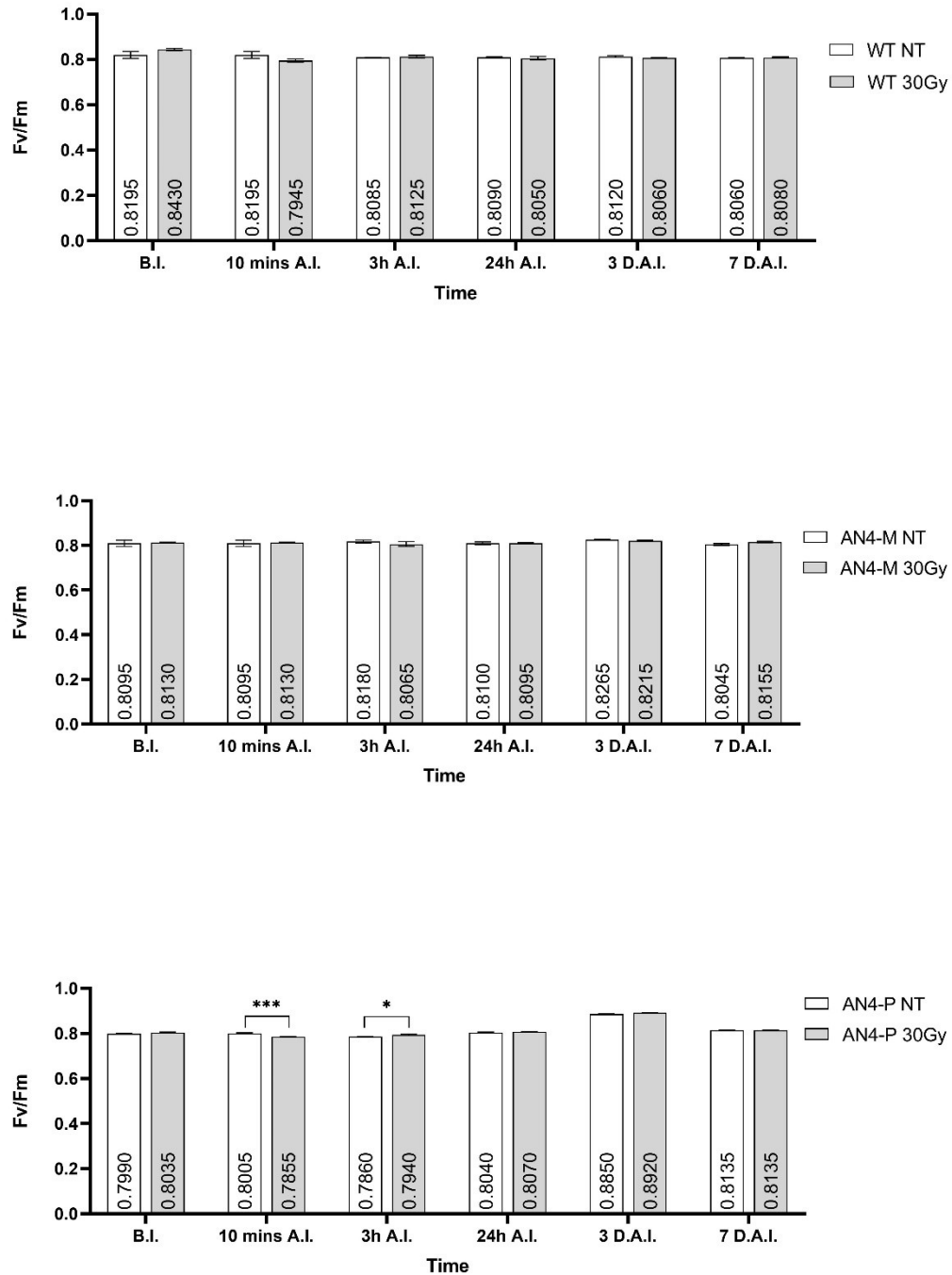


Figure 4. 95 Analysis of the Fv / Fm index of wild type, AN4-M and AN4-P₂ MicroTom along the observation period upon different irradiation treatments supplied to plants at 45 DAS. Statistical relevance: *: p<0.05; **: p<0.001; ***: p<0.0001.

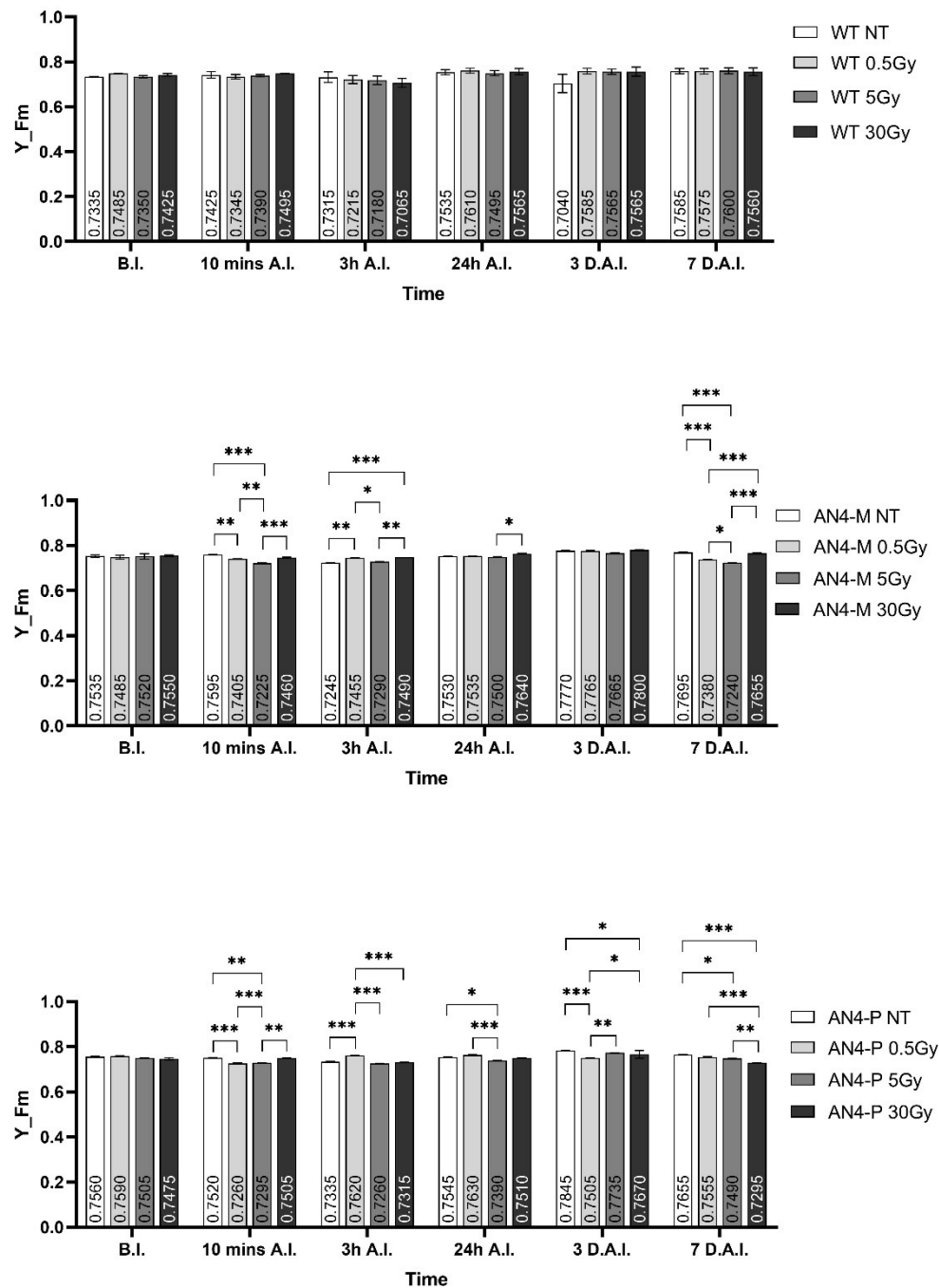


Figure 4. 96 Analysis of the Y_Fm index of wild type, AN4-M and AN4-P₂ MicroTom along the observation period upon different irradiation treatments supplied at 45 DAS. Statistical relevance: *: p<0.05; **: p<0.001; ***: p<0.0001.

5. DISCUSSION

Designing plants for future space manned missions

There is rising interest in the development of biofortified plants that can cope with outer space conditions due to accumulation of natural antioxidants to be introduced with the diet of astronauts. Among the secondary metabolites providing plant protection against environmental challenges, anthocyanins are well-known for their potent anti-oxidant properties. These compounds are candidate protectants against cosmic radiation to be introduced in the diet of astronauts during spaceflight, as well (Gómez et al., 2021). Plant biotechnology methods may ~~thus~~ be exploited to boost biosynthetic pathways for the generation of plants capable to deal with non-terrestrial conditions and to improve astronauts' health.

Solanum lycopersicum cv. MicroTom was chosen due to proven suitability to agriculture in the confined area of a crewed space module (Dueck et al., 2016). In particular, MicroTom plants aimed at 'space evolution' of tomato were engineered through the ectopic expression of the anthocyanin R2R3-MYB transcription factor *PhAN4* from *Petunia hybrida*. *PhAN4* belongs to the SG6 MYB factors, like *SlAN2*, *SlANT1* and *SlAN2-like*, which overexpression in tomato functions in upregulating anthocyanin biosynthesis. SG6 MYBs may also regulate other processes related to agronomical traits of interest, as it was already specifically demonstrated for *SlAN2* (i.e., upregulation of flavour- and ripening-related genes, beside the phenylpropanoid pathway). In the design of a space MicroTom, we used a *PhAN4*-single gene approach to possibly impact different traits, among which ~~possibly~~ biotic/abiotic/oxidative stress resistance ~~ones~~. Unlike other tomato MYBs that have been studied mainly through their over-expression, *PhAN4* has been deeply characterized also based on loss-of-function mutants (Spelt et al., 2000; Povero, 2011).

The MicroTom PhAN4 HRCs model demonstrates the restoration of bioaccumulation of anthocyanins and other polyphenols in tomato cells, their high stability upon strong pro-oxidant stress and the regulation of resistance traits by PhAN4

To initially understand the *PhAN4* gene function in tomato, hairy roots cultures (HRCs) were generated as a simplified model before whole plants transformation (Massa et al., 2022). The response to the constitutive expression of *PhAN4* in tomato HRCs was analyzed transcriptome-wide by RNA-seq analysis and compared to control HRCs. RNA-seq depicted a reprogramming oriented not only to anthocyanin accumulation, as expected, but also to positive regulation of fruit quality-related genes, and cell response to biotic, abiotic and oxidative stress, as well.

With the exception of *PAL*, *C4H*, *4CL*, *F3'H*, and *5UFGT*, the other structural genes of the anthocyanins pathway were up-regulated. *CHS* and *CHI* had been already found to be up-regulated by overexpression of *SLAN2* in tomato, but not by ectopic expression of *Del/Ros* that, in turn, were able to upregulate *F3H* (Butelli et al., 2008; Jian et al., 2019). *DFR* had been already shown to be up-regulated in the stem and leaf of tomato plants overexpressing *SLAN2* and *SLAN1* (Kiferle et al., 2015). Interestingly, despite we could not find *FLS* among up-regulated DEGs in AN4 HRCs, *LDOX*, a known bi-functional enzyme being able not only the conversion of leucoanthocyanidins in anthocyanidins, but also to catalyze the formation of flavonols, resulted to be differentially up-regulated in AN4 HRCs. Interestingly, *DilFl* a tomato homologue of cytochrome b5 of *P. hybrida*, essential for full activity of F3'5'H, was found to be up-regulated in AN4 HRCs. Among late biosynthetic genes, up-regulation of *ANS* had been already shown upon *SLAN2* over-expression and *Del/Ros* expression in tomato as it was in our AN4 HRCs. *OMT* and *AAT* transferases have already been described to be targets of a MYB in tomato, as well (Zhang et al., 2019). Their up-regulation in AN4 HRCs, together with that of *GST*, *PH3*, *OMT*, *3UFGT*, *RT* and *AAT* may explain the accumulation of the glycosylated and acylated forms of anthocyanidins. As tomato lacks PH1 and PH5, two P-ATPases that in *Petunia* cooperates with PH3 and PH4 in determining flower color by hyperacidification of petal cell vacuoles, *PH3* (Solyc10g084380.1.1) could be associated with anthocyanin biosynthesis boosting, than acidification of pH. The up-regulation of anthocyanin biosynthesis-relevant transcription factors further expands the influence of AN4 MYB in tomato gene expression regulation. Among those, the found up-regulation of *SLAN1*, *SLAN2* and *WD40* is coherent with already proposed models of anthocyanin biosynthesis regulation (Koes et al., 2005; Liu et al., 2018).

Interestingly, the *PhAN4*-related transcriptomic enhancement was found to involve more than 30 genes specifically related to response to biotic/abiotic and oxidative stress, as well. Indeed, GOE analysis was associated to proteinase inhibitors, beta-glucosidases, glucanases, glucosylases, xylan acetylases, resistance proteins and other classes of enzymes that are players of defence against pathogens and pests and of response to abiotic stresses specifically associated with wounding, cold, heat, drought, hypoxia, and to UV exposure in tomato (Fan et al., 2020). Among those, the up-regulated beta-glucosidases, together with the dirigent proteins and the monoterpene and sesquiterpene synthases that were found, are involved in the formation of required intermediates for cell wall lignification. The positive regulation of trichome birefringence-like protein mediating xylan acetylation, is also another tool in possible protection against environmental stresses among which there is cold and excess of minerals in soil. Acetylation of wall polymers is, indeed, vital for plant growth and adaptation to various

environments, and is required for structural integrity of the leaf surface exerting a global impact on plant stress responses (Gao et al., 2017). In relation to the positive regulation of beta-1,3-glucanases, beta-glucosidases and xylan acetylases, together with expansins, it has to be added that these genes are related also to fruit softening or improved emission of volatiles (Tzin et al., 2015; Li et al., 2020b). In this sense, the positive regulation of chlorophyll a/b binding protein (that is normally up-regulated during fruit ripening) and of one gene related to volatile compounds biosynthesis (*chorismate mutase*) was found in AN4 HRCs. On the contrary, P450, that is involved in extended shelf life of fruits, was found to be down-regulated.

One player of ROS detoxification and regulator of the redox signaling network of tomato, plastidial thioredoxin Y2, was found to be highly up-regulated, as well. Moreover, the RNA-seq analysis demonstrated the down-regulation of violaxanthin de-epoxidase (*VDE*) and carotenoid isomerase (*CRTISO*), two genes involved in the carotenoid biosynthesis. Despite being involved in the photo-inhibition of the PSII, suppression of VDE can induce, at the same time, an accumulation of fucoxanthin that functions as an efficient anti-oxidant in anoxia conditions (Mikami and Hosokawa, 2013). The down-regulation of carotenoid isomerase was demonstrated to induce an accumulation of zeta-carotene and cis-prolycopene in tomato fruits (Isaacson et al., 2002; Pinheiro et al., 2019), both elevating and modifying carotenoid profiles towards more bioavailable forms compared to wild-type (Cooperstone et al., 2015). Hence, the ROS counteracting potential of AN4 HRC material may be related not only to anthocyanins but also to other accumulating antioxidant specialized metabolites.

Transcriptome-wide RNA-seq data was supported by the accumulation of anthocyanins and phenylpropanoids in HRCs obtained by mass spectrometry. In accordance with literature related to transgenic tomato plants (Butelli et al., 2008; Su et al., 2016; Jian et al., 2019), mass spectrometry allowed us to identify glycosylated and acylated delphinidin and petunidin as the most abundant anthocyanins in AN4 HRCs. Interestingly, two additional anthocyanins, unusual in tomato, pelargonidin-3-glucoside and malvidin-3-O-(4''coumaroyl)-rutinose-5-O-glucose, were detected, as well. Malvidin and Pelargonidin derivatives have already been detected in Del and Ros1 and in LC and C1 tomato plants, respectively (Bovy et al., 2002; Su et al., 2016). Interestingly, pelargonidin plays a major role in reducing genotoxic stress induced by environmental toxicants in plants (Khandelwal and Abraham, 2014). In addition, rather than other anthocyanins, pelargonidin-3-glucoside, that was accumulated in engineered MicroTom HRCs, has high bio-availability, being absorbed in an intact form into the gastrointestinal wall, undergoing first-pass metabolism and entering the systemic circulation as 4-hydroxybenzoic acid, a stable metabolite that is considered one of the main players of the systemic health effects

of anthocyanins (Fang, 2014). In order to achieve a better understanding of *PhAN4* in tomato hairy root phenylpropanoid pathway, we measured 13 phenolic acids and derivatives and 29 flavonoids: notably, a reduction in the content of some members of the former, and a massive increase in most of compounds in the latter were observed, thus proving *PhAN4* is able to trigger the metabolic flux at both flavonoid and anthocyanin levels.

In view of the future development of natural anti-oxidants produced by engineered plants and possibly administered with the diet, and to define whether the significantly higher DPPH antioxidant capacity of specialized metabolites accumulating in AN4 HRCs may be efficiently maintained after a strong pro-oxidant stimulus, we exposed lyophilized biomass from AN4 HRC, to high dose ^{60}Co gamma radiation. Gamma rays are a component of cosmic ionizing radiation and induce the formation of free radical species (i.e., paramagnetic species). Gamma rays are perfect ROS inducers both directly through water radiolysis, and indirectly via the activation of a broad range of signalling processes (e.g., damages to the mitochondria or cell microenvironment) (Azzam et al., 2012). ESR Spectroscopy was used as a sensitive tool to identify the entity of the paramagnetic species generated in the HCRs samples after gamma irradiation (D'Errico et al., 2018). ESR analyses revealed a significant difference in the overall amounts of radical species accumulated in the two HRC molecular backgrounds. At the same absorbed dose, control generates a very intense peroxy radical signal, while a considerably lower level of the singlet intensity is generated in AN4. This reveals that the molecular set formed upon *PhAN4* expression provides very effective free radical scavengers efficiently counteracting oxidative stress upon gamma radiation.

The UV-VIS analysis allowed us to confirm the results obtained by metabolomic data and to characterize the effect of gamma rays on the anti-oxidant compounds. In fact, gamma irradiation, via releasing free radicals in solution, may alter these plant constituents (Lalande et al., 2019). Compared to the substantially unchanged spectra of AN4 HRCs, the absorbance decrease of the 320 nm peak (i.e. flavonols, hydroxycinnamic acids, tannins, flavanols and anthocyanins associated to caffeic and coumaric acids) in controls upon irradiation, suggests that the anthocyanins accumulated in AN4 HRCs are particularly stable to gamma high absorbed dose and probably have protective effects on other biomolecules. Indirectly, this result shows that these molecules may give the major contribution to the ROS buffering capacity under radiation shown by ESR and to the overall antioxidant potential. As a confirmation of the possible significance of this result *in vivo*, it was reported that, even at higher absorbed doses than that used in this work, X rays keep the level of flavonoids in aqueous solution unchanged due to the

radiolysis-mediated formation of depsides that, in addition, maintain good anti-oxidant properties, as well (Lateef and Al-Nimer, 2009).

For proteins to be functional within a cell requires coordinated folding processes to obtain a correct 3D shape. Disruptions to protein folding, that can occur in space due to ionizing radiation, can have profound biological implications for all organisms, including plant cells, leading to dysfunctions (Blanco et al., 2018). For this reason, investigations were conducted through photoluminescence analysis on HRCs total soluble protein extracts. When a protein is exposed to the wavelength of 280 nm, mainly the tryptophan and tyrosine residues get excited, which would reflect upon its tertiary structure (Yang et al., 2015a). The maximum and emission peak position reflects upon secondary, tertiary and quaternary structure (Hilaire et al., 2017). Moreover, the extent of protein oxidation is measured by determining the loss of specific tryptophan fluorescence. The emission spectra of HRCs showed an overall decrease in maximum emission intensities only in the case of irradiated controls, revealing that structure of soluble proteins is partially lost upon irradiation in those samples. This result demonstrates a low capacity of proteins of control HRCs to counteract unfolding, binding to hydrophobic pockets and aggregation, witnessed by the loss of fluorescence due to the burial of tryptophan residues after radiation. In particular, the $\Delta\%$ photoluminescence intensity / mass units after and before irradiation, indicated that soluble proteins from control undergo a doubled oxidative stress compared to AN4 samples. Being polyphenols emitting at 280 and 320 nm extracted by PBS together with total soluble proteins, contribution of these classes of molecules may be hypothesized in stabilizing proteins, as proofs of interaction of anthocyanins with proteins seem to suggest (Sui et al., 2018). Possible additional contributions of enzymatic players to mitigation of misfolding and aggregation *in vivo* may be object of future studies.

Summarizing, MicroTom HRCs allowed to rapidly test *PhAN4* expression cassette on tomato cells, opening the way to application to engineering of whole plants able to perform in a suitable and predictable manner.

The engineered MicroTom PhAN4 plants show favourable features in terms of size, productivity, accumulation of anthocyanins, antioxidant properties

The whole *PhAN4* MicroTom plant generation was accomplished through *Agrobacterium*-mediated transformation of wild type. TLA, commonly used for samples of animal origin, was applied to MicroTom plants as a faster and cheaper approach compared to whole genome sequencing (e.g., CONTRAILS described by Lambirth et al., 2015) usually applied to plant

samples. TLA allowed to confirm zygosity and insertion site of the engineered plants, assuring no significant SNVs and with no interruption of relevant gene sequences.

The homozygous AN4-M and the hemizygous AN4-P₂ plants, were chosen for subsequent characterization. In particular, the hemizygous AN4-P₂ plants showed the most favourable features related to anthocyanins accumulation compared to both wild type and homozygous AN4-M line. This finding is in accordance with observations of Butelli et al., 2008 for a fruit-specific purple phenotype induced by the expression of *Delila* and *Rosea1* in MicroTom and that was, for this reason, transferred to other genetic backgrounds by crossing to take advantage of it. The phenotypic characterization of the AN4 plants revealed that life-cycle and yield traits were or were not positively influenced by the presence of the *PhAN4* transgene, respectively. In addition, as a pleiotropic effect, engineered plants showed a miniaturized habitus compared to wild type. This behaviour may be explained considering the secondary metabolites-oriented production driven by *PhAN4* at the expense of primary metabolism. This effect, combined with the overall conserved productivity of the engineered plants, points to a plant ideotype with beneficial features in view of the confined environments of spacecrafts and outposts (Stutte et al., 1999; Paradiso et al., 2014).

As reported by Cerovic G. et al., 1999, the fluorescence signals combined with a characteristic fluorescence signature provide valuable information about the type, localisation and concentration of specific fluorophores in plant tissues. Hence, changes in the fluorescence signature, caused by alterations in the amount and composition of fluorescing pigments, might be used as indicators to study plant physiology (Lichtenthaler et al., 1998).

SFR and NBI indices detected using Multiplex® sensor FORCE-A, showed a sort of periodic sigmoidal-like trend for all genotypes but with different periodicity. While the SFR index is positively correlated with the chlorophyll content, the NBI index can provide a direct *in vivo* information on metabolism status and it is considered a better indicator of C/N allocation changes than a measurement of leaf chlorophyll content *per se* (Ronga et al., 2018). In our experimental conditions, AN4-M and AN4-P₂ transgenic plants anticipate by one week and two weeks, respectively, the first peak of NBI compared to wild type. This could be due to an earlier decrease of the secondary metabolism level compared to wild type, as confirmed by visual inspection of transgenic plants, showing a sort of attenuation of general pigmentation in correspondence of these moments and in accordance with the trend of the FLAV index that is related to the accumulation of epidermal flavonols (Bilger et al., 1997).

It is known that anthocyanins, which trend we followed using the ANTH index, act as ROS-scavenging compounds that participate to both enzymatic and non-enzymatic antioxidant

mechanisms (Ullah et al., 2019). The ANTH values were constant and negative throughout the life cycle of wild type, indicating absence of anthocyanins, while both AN4-M and AN4-P₂ were characterized by a sigmoidal curve with a main inflection point between 44 and 51 DAI (44 -51 DAS) that reflects the shift from the vegetative state to the flowering time.

Despite the decrease of anthocyanins content at the red ripe stage, the increasing antioxidant capacity of engineered fruits along ripening stages, points to the possible additional contribution of other antioxidant metabolites like vitamin C and E, and carotenoids, reaching progressively maximum accumulation at the ripe stage. The statistical equivalence or superiority of the engineered ripe fruits antioxidant capacity compared to ripe wild type ripe fruits can be explained by a possible cross talk between the anthocyanins and the carotenoids pathways, leading to improvement of carotenoid levels. Indeed, transcriptomic analysis of tomato *PhAN4*-expressing HRC cells has revealed down-regulation of violaxanthin de-epoxidase and carotenoid isomerase, both biosynthetic genes leading to carotenoid very bioavailable forms accumulation (Massa et al., 2022). The improvement of the antioxidant capacity in engineered AN4-P₂ plants compared to wild type and AN4-M, may also point to the possible additional contribution of ROS-scavenging enzymatic players such as glutathione peroxidase, catalase, and superoxide dismutase may be involved. Interestingly, it has been demonstrated that anthocyanins interact with these enzymes during scavenging processes (Salehi et al., 2020). In addition, transcriptomic analysis of tomato *PhAN4*-expressing HRC cells has revealed that *PhAN4* upregulates plastidial thioredoxin Y2 that is a player of ROS detoxification and involved in the redox signalling network of tomato (Serrato et al., 2013). Preliminary transcriptomic evaluation on AN4-P₂ ripe fruits seems to corroborate this hypothesis.

To evaluate the potential of *PhAN4*-biofortification to protect humans (as the basis of antioxidant space food) and plants themselves from oxidative damage we evaluated, through ESR and photoluminescence analysis, the fruits' differential capacity to counteract the generation of ROS and preserve protein folding. As expected, by exposure to an equal dose of radiation, AN4-P₂ and AN4-M ripe fruits' metabolite content have a higher radical scavenging ability compared to wild type, resulting in a lower level of radicals accumulated. The ESR signal analysis showed that the two main peaks are attributable to the radiation-induced cellulose radicals, as predicted for a plant matrix (Duliu and Bercu, 2017).

In addition, the high dose gamma irradiation did not result in any detectable decrease in fluorescence emission intensity, demonstrating a general resistance of MicroTom fruit soluble proteins' tertiary and quaternary structure to this stress. Nevertheless, the blue shift phenomenon observed at a lesser extent for AN4-P₂, demonstrated that the soluble proteins of these fruits have

side chain indole of tryptophan that are less prone to undergo oxidation upon strong gamma radiation than wild type and AN4-M.

Evaluating plant response to the gamma radiation component of IR

Exposure to ionizing radiation, at doses that plants would possibly experience during different hypothesized scenarios of a long-term space mission, was reproduced on a lab scale using ground-based facilities. The setup of ground-based chronic exposure experiments represents a major issue due to logistical challenges and acute irradiation remains the most approachable method to study the effects of space radiation on plants. Therefore, we supplied acute irradiation treatments to engineered tomato seeds and to plants at two developmental stages (30 and 45 days after sowing). Several studies have demonstrated that plant response depends largely on the type of radiation, its dose and its dose rate (Caplin and Willey, 2018). Furthermore, species, cultivar, plant age (seed or seedling), organ, irradiated tissue and ploidy level can also influence plants responses (Micco et al., 2011). Survival of plants depends on the nature of DNA damage, which increases with increasing doses of radiation (Pick and Ling, 2008). IR generally affects offspring plant physiology and morphology, with consequences also on growth development and reproduction success (Rea et al., 2008). In some cases, IR can influence the content of phenolic compounds (Arena et al., 2014b; De Micco et al., 2014) or the photosynthetic apparatus at different levels (i.e., electron transport carriers, light-harvesting complexes, carbon reduction cycle enzymes and chlorophyll a and b) (Kim et al., 2004; Marcu et al., 2013b). Considering all these different key factors, it is difficult to depict a concise plant response behaviour, although general considerations can be made.

Despite some studies have been conducted on seedlings of several species (i.e., *Pisum sativum*, *Phoenix dactylifera*, *Lactuca sativa*, *Arabidopsis thaliana*, *Zizania latifolia*) (Zaka et al., 2004; Kurimoto et al., 2010; Kim et al., 2011; Al-khayri, 2012; Marcu et al., 2013a; Arena et al., 2014), little is known about the effects of cosmic rays on "adult" plants, and, currently, no experiments have been reported on fully developed tomato plants. We adopted two approaches to monitor the plants irradiation response: a biometric and a fluorimetric methodology. These approaches can give two types of information: biometric parameters reflect the changes occurred at both phenotypic and phenological level, non-destructive fluorimetric measurements indirectly reflect the changes occurred at physiological level.

Offspring of engineered MicroTom PhAN4 irradiated seeds show very limited response to this pro-oxidant stimulus

Despite the wealth of literature regarding treatments with gamma rays, in our knowledge this is the first time that ^{60}Co radiation are supplied to tomato seeds and plants aimed at simulating space cultivation conditions. Indeed, to date, De Micco et al., 2014 and Arena et al., 2019 used low-LET radiation (X rays from 0.3 to 100 Gy dosages) and high-LET radiation (Ca ions with 25 Gy dose), respectively, on tomato seeds. Due to differences related to the radiation source and type of radiation is not possible to make complete comparison with these two studies.

The characterization of offspring of irradiated seeds revealed statistically significant variations only in relation to a very limited number of parameters. In particular, only yield traits while no changes occurred for other parameters, demonstrating that gamma rays up to 30 Gy do not induce structural aberrations responsible for lowering physiological performance of MicroTom plants, as already found by Arena et al., 2019. Despite the three genotypes have statistically similar control values of these parameters (i.e., in absence of irradiation), irradiation of seeds exerted a differential effect depending on genotype, with significantly positive variations of the number of seeds for both wild type and AN4-M offspring. These data give an initial hint that the specific genotype has had an influence on the final effect and that, in this specific experimental setup, the AN4-M plants performed very well in terms of response to gamma rays, showing favourable characteristics for seeding in space in different conditions (see Figure 3.1 in methods section). Coupling this response to the ability to accumulate anthocyanins and general smaller size compared to wild type, makes the outcome even more favourable for AN4-M seeds.

In our knowledge, this is the first time that the Multiplex Force A™ sensor is employed to evaluate the suitability of the multiparametric fluorescence technique as a non-destructive tool for monitoring plants response to gamma rays during an entire cultivation trial. At the end of the experiment, plants obtained from irradiated seeds showed no statistical variation of the considered indices, demonstrating that offspring of irradiated seeds are able to eventually tackle gamma rays through adjustments that occurs during the development period. Conversely, different response to irradiation were detected in function of the genotypes throughout the detection period. We found that gamma rays caused an increase of the SFR index that, in literature, is generally recognized as an indirect effect of the increase of proline that contributes to improve the stability of chlorophyll molecules, to scavenge ROS as well as to stabilize membranes (Kautz et al., 2014). In addition, Kim et al., 2011 and Micco et al., 2014 described the chlorophyll increase as a consequence of changes in number and sizes of chloroplast. Conversely, the decrease of the SFR index, that we found for wild type plants subjected to the

0.5 Gy dose at 51 DAI (51 DAS), was described by Dartnell et al., 2011 as a result from ROS-mediated oxidation of the pigments. Interestingly, the significant decrease of the NBI index, as found in wild type plants (51 DAI) and in AN4-P₂ plants (37 and 44 DAI), or its increase (at 30 DAI in AN4-M plants) depends on the nitrogen metabolism and, in particular, on the reallocation of nitrogen compounds from older to young leaves to enhance abiotic stress tolerance (Vijayakumari et al., 2016; Volkova et al., 2020).

Moreover, the main gamma rays target is recognized to be the photosystem II, which is the pigment-protein complex in the chloroplast. In accordance with values detected for SFR and NBI indices, the photosynthetic maximum photochemical efficiency (Fv/Fm) and the maximum fluorescent yield (Y_{Fm}) resulted in the range described for healthy leaves by the Plant Stress Kit (PSKII) manufacturer (0.7/0.9), indicating no alteration by irradiation of the performances of the photosynthetic apparatus and its capacity to recovery from this stress. Similar results have been reported among *Solanaceae* on potato and red pepper subjected to gamma rays (Kim et al., 2002, 2004). Even though within the ranges, some statistically relevant differences were found at the end of the detection period. As reported by Arena et al., 2019, these changes in photochemical performances relies on changes that occurs on the PSII D1, where electron transfer between the primary electron donor, Tyr Z, and the secondary plastoquinone acceptor, QB, occurs.

It is well proven that the biosynthesis of flavonoids is up regulated in response to abiotic stresses and to IR, as well (Di Ferdinando et al., 2012). As reported by Agati et al., 2011, changes of the FLAV index are generally attributable to the ortho-dihydroxylated B-ring of quercetin-3-O- and lutein-7-O-glycosides. In accordance with literature, gamma rays constantly resulted in an increase of the FLAV index in our experimental setup (Kova, 2002; Vardhan and Shukla, 2017). In particular, especially the 0.5 and 5 Gy dosages, produced an FLAV index-related early response (between 37 and 44 DAS) in AN4 plants, and a late FLAV index-related response (58 DAS) in wild type. The different temporal response registered by the FLAV index between AN4 and wild type plants may be due to the effect of *PhAN4* gene that strongly turned on the entire phenylpropanoid biosynthetic pathway, making engineered plants more responsive in the production of these secondary metabolites than wild type.

The ANTH index resulted poorly affected in all the three genotypes. This is not surprising for wild type plants since they are not able to synthesize anthocyanins. This result in AN4 plants demonstrates that gamma rays supplied to seeds were not able to interfere with the accumulation of anthocyanins in engineered offspring. As somehow expected, we found that AN4 plants are

more similar each other in relation to the value trend of indices related to the flavonoids (FLAV and ANTH), as they constitutively express the *PhAN4* gene.

Data previously reported for acute gamma irradiation of MicroTom seeds had already pointed out that seeds are more resistant than other phenological stages. This can be explained considering their structure, their low H₂O content and the high levels of compounds like melatonin that could play an important role in the antioxidant defence through the protection of the endosperm and the reproductive tissue (Arena et al., 2019a).

MicroTom PhAN4 plants would not complete a seed-to-seed cycle under conditions mimicking an exceptional solar flare event at 30 DAS (like wild types), but would cope with different scenarios related to GCRs and SEPs during cultivation on Mars surface mounting no or beneficial responses

In order to fill the lack of knowledge about the effects of cosmic rays on fully developed tomato plants, wild type and AN4 plants at two developmental stages (30 DAS and 45 DAS) were subjected to gamma irradiation. The 30 DAS life stage represents the crucial period when MicroTom has completed 30% of its entire life cycle and the first true leaves are fully developed. At this life stage, MicroTom is almost ready to initiate the flowering time, that is pivotal for subsequent fruit set.

As highlighted for experiment conducted on seeds, also treatments supplied to plants at 30 DAS resulted in different responses among genotypes. At the end of the measurement period, we found that irradiation caused some visible phenotypic effect. In particular, the higher number of leaves and leaf area can be considered a detrimental effect in view of optimizing space while maintaining the same yield. Conversely, the significantly lowering of height and number of leaves of AN4-P₂ plants irradiated at 5 Gy can be considered as beneficial traits for cultivation processes in a confined environment. In addition, we found that the AN4-M plants performed very well in terms of response to gamma rays, producing a higher number of seeds upon treatments if compared to wild type and AN4-P₂ plants. To date, the physiological and biochemical processes that determine changes in growth and development of plants subjected to IR remains unknown (Gudkov et al., 2019).

Irradiation at 30 Gy determined an overall, significant negative effects on both vegetative and yield traits independently from the genotype, with drastic loss of the number of flowers and consequences on number of fruits and fruits-related parameters, that was not observed in offspring of irradiated seeds. This may be related with the fact that the 30 DAS life cycle stage is crucial for completing the entire life cycle since it coincides with flowering time. Moreover, it

can be concluded that, between 5 and 30 Gy, there is limiting dose that tomato plants at 30 DAS can't handle.

Fluorescence indices were differently influenced in dependence of irradiation dose supplied to the different genotypes. At the end of the measurement period, the SFR decrease found in AN4 plants subjected to the 30 Gy dose reflected the detrimental effect of this radiation dosage on morpho-anatomical traits. Also the NBI index was influenced by the irradiation. In particular, wild type plants subjected to the 5 Gy dose showed an increase of the NBI index that is generally attributed to changes in sugar interconversion (i.e., sugar content increases in plants subjected to IR at low doses (Ahuja et al., 2014)). Coherently with the radiation-induced stress and the responsiveness conferred by the *PhAN4* gene, AN4 plants, and in particular AN4-M plants subjected to the 5 and 30 Gy dose and AN4-P₂ plants subjected to the 30 Gy dose, constantly resulted in an increase of the FLAV index. In addition, the irradiation treatment (5 and 30 Gy) supplied to AN4-M produced a slight increase also on the ANTH index while, interestingly, wild type plants subjected to the 5 Gy dose and AN4-P₂ plants subjected to the 0.5 Gy dose experienced a decrease of the ANTH index. This decrease can be associated with the polyphenolic structure of anthocyanin, which are prone to oxidation and vulnerable to oxidative degradation. As an example, previous studies proposed that anthocyanin bleaching is the result of the condensation of ascorbic acid at carbon-4 (C4) of the anthocyanin as this site is the most susceptible to electrophilic attack (Farr and Giusti, 2018).

Differently from seedlings of irradiated seeds, the whole of fluorimetric indices values detected along the measurement period in plants irradiated at 30 DAS allowed to depict an early and a late overall stress response. The bi-phasic response of plants to stress induced by acute irradiation is a well-known mechanism (Gudkov et al., 2019). The first phase is a short-term, non-specific stress response which is a generic reaction of the plant to an intense stimulus, as that found for abiotic and biotic stresses. The second phase, which is characterized by an increased ROS level, appears a few days after the irradiation (approximately around 14 DAI in our experimental condition) and is largely associated with the cumulative damages to the structural components of the cell and, as a result, to disruption of the normal functioning of many processes (as in the case of the 30 Gy effect in our experimental condition). Interestingly, during the first phase, the SFR values increased only in wild type (0.5 Gy at 3h AI) and in AN4-P₂ plants (5 Gy at 3h AI), while the second phase (from 28 to 49 DAI in the case of the SFR index) resulted in a decrease of the index in AN4 plants subjected to the 30 Gy dose and in a slight increase for AN4-M irradiated with the 0.5 or 5 Gy dose. Conversely, in relation to the NBI index, the first phase of response to gamma rays was found only in AN4-P₂ plants that, when subjected to the 5 Gy dose, showed

changes that resulted in an increase of the index straight after the irradiation procedure until 24h AI. The NBI index well intercepted the second phase of the irradiation response. In fact, gamma ray treatments, and especially the 30 Gy dose, resulted in a decrease of the index for both AN4-M and AN4-P₂ plants starting from the 21 DAI (51 DAS) until the end of the detection period. Interestingly, in wild type plants subjected to the 5 Gy dose a slight decrease of the NBI index was observed at 35 DAI (65 DAS) followed by an increase during the two last measurements, underlying an opposite behaviour of wild type compared to engineered genotypes.

Wild type plants, probably due to the low content of flavonoids, lacked the first phase of the response while the second phase resulted mild visible between 35 and 42 DAI (65 and 72 DAS). Conversely AN4-M plants, probably due to the consequences of the expression of the *PhAN4* gene, showed a strong and constant increase of the index starting from 24h AI until the end of the measurement, making the two phases of response indistinguishable. Only AN4-P₂ plants showed a biphasic behaviour: the FLAV index decreased straight after the irradiation (0.5 and 5 Gy dose effect at 10 min AI) and at 14 DAI (44 DAS) (5 Gy effect), increasing from 28 to 49 DAI (58 to 79 DAS). This behaviour can be explained considering that flavonoids in AN4-P₂ plants are more available (due to their higher level compared to the other genotypes) to enzymatic players that use phenolic compounds as electron donors. In fact, during the first 3-4 hours after irradiation, the H₂O₂ generated inside chloroplasts, mitochondria or peroxisomes generally diffuses into the vacuoles to be scavenged in reactions catalysed by class-III peroxidases (Samson et al., 2019). The amount of flavonoids in AN4-P₂ plants subjected to 0.5 and 5 Gy doses is probably able to counteract the gamma ray-related stress, while the increase of the FLAV index during the second phase of the response upon the 30 Gy dose, reflects the need to produce antioxidant compounds continuously. The strong response to gamma rays supplied to plants at 30 DAS was also visible through the ANTH index that constantly increased during the measurement period, especially for plants irradiated with the 30 Gy dose. In this case, the two phases of response to IR were indistinguishable for both wild type and AN4-P₂ plants. Conversely, only the second phase of the response resulted detectable in AN4-M plants subjected to the 30 Gy dose starting from 21 DAI (51 DAS). From 14/21 DAI (44/51 DAS) both wild type and AN4 plants were experiencing the effect of gamma rays at phenotypic level (leaf area and number of flowers were already compromised) and the ANTH index well reflected this scenario at physiological level. As discussed for data obtained from irradiated seeds, photosynthetic performances were not influenced by the irradiation and only slight differences were attributable to changes that occurs on the PSII D1 expression (Arena et al., 2019). Comparing the results obtained from irradiated seeds and plants at 30 DAS it is clear that gamma rays differently affect

both biometric and fluorimetric parameters. The differences found can be ascribed to the different water content of the two phenological stages. In fact, it is well-known that a significant part (about 70-80%) of the IR-related DNA damage is caused by ROS formed during radiolysis of water (Gudkov et al., 2016). Hence, if compared with seeds, the greater amount of water content in fully developed plants favours the radiolysis process, generating more ROS and subsequently a higher level of DNA damage. In accordance with data obtained from irradiated seeds, no changes occurred for AN4-M plants subjected to the 0.5 and 5 Gy dose on both vegetative and yield traits, confirming that this genotype is more capable to counteract the radiation stress also at this phenological stage compared to AN4-P₂. Moreover, fluorescence-based indices demonstrated that, even if changes occurred during the measurement period for both phenological stages, these were more pronounced in plants irradiated at 30 DAS, as strongly reinforced by data obtained at the end of the measurements. In particular, only AN4-M plants irradiated at 30 DAS showed changes of the considered indices, demonstrating that this genotype can better counteract IR effects due to physiological adjustments that make it more tolerant to this stress.

Offspring of plants irradiated at 30 DAS resulted affected by parental ionizing radiation exposure, but inheritance of parental mutations should be excluded

Kordyum and Hasenstein, 2021 recently pointed out that there is limited information regarding the possible effect of IR on subsequent generations of plants. Until 2004, only Zaka and colleagues studied the effects of gamma rays on two generations of *Pisum sativum* seedlings, which resulted in genomic instability and consequential smaller plants. One of the most relevant work has been conducted by Yu et al., 2007 on board the Shenzhou-3 spaceship. This experiment, conducted on three generations of rice, revealed that mutation traits can be inherited by the progeny. Following this line, during our work, seeds obtained from plants treated at 30 DAS were planted and both morphometric and fluorimetric measurements were conducted.

Leaf area was positively influenced in all the three genotypes, while number of leaves and number of fruits were positively affected only in AN4-M plants. These changes became visible only during the measurement conducted at 60 DAS and were maintained until the end of the measurements, demonstrating that plants developed normally during the first part of their life cycle. For both 0.5 and 5 Gy dose, no detrimental effects were detected on other yield traits, as seen in the irradiated parental lines. In particular, as above reported for offspring of irradiated seeds and plants at 30 DAS, a higher number of leaves and leaves area can be considered detrimental in view of optimizing space. On the contrary, the increase in the number of fruits, as

in the case of AN4-M plants, is a desired trait. To be noted that the significantly different parameters found in the offspring were not the same of the parental genotypes, demonstrating that these changes were not inherited as mutations as reported by Yu et al., 2007, but can be ascribed to other changes (i.e., decrease in HR frequency or epigenetic changes) (Kovalchuk et al., 2004). However, it is worth to note that experiments conducted by Yu et al., 2007 were performed after a spaceflight mission in which plants were also subjected to microgravity and this stimulus could influence in a different manner the repair processes and the way plants inherits traits. As reported in relation to the biometric parameters, the changes occurred for fluorescence-based indices, were not the same encountered in plants irradiated at 30 DAS, with the only exception of FLAV and ANTH indices for AN4-M plants obtained from plants irradiated with the 5 Gy dose. As described by Kovalchuk et al., 2004, offspring of irradiated plants could experience changes in expression of important housekeeping and DNA-repair genes that could possibly lead to stronger plants, more capable of tackling environmental challenges. At the end of the measurements, we found some changes in values of the indices considered, demonstrating that transgenerational regulation of genes involved in stress response occurred, coherently with data obtained from *Arabidopsis* studies (Walle et al., 2016). Along the measurement period, the two phases response to IR were not detectable and only a second phase-similar response was found (especially for FLAV index, independently from the genotype, starting from 30 DAS). As seen for offspring of irradiated seeds and plants irradiated at 30 DAS, no changes were found in photochemical performance indices also in the offspring of irradiated 30 DAS plants, confirming that the photosynthetic apparatus maintained a healthy status. Summarizing, some consequences of parental irradiation were observed in offspring. These consequences are probably not only related to the damage of key cellular structures, but also to regulation at genetic level. These changes can be caused by long-term changes via hypermethylation and altered frequency of transitional recombination (Walle et al., 2016).

MicroTom PhAN4 plants would not complete a seed-to-seed cycle mimicking an exceptional solar flare event at 45 DAS (like wild types), but would decrease the capacity to modulate stress response compared to younger plants

Lastly, to evaluate if changes different from those observed in plants at 30 DAS may occur if plants are challenged at later stages, we analyzed plants irradiated at 45 DAS. This life stage represents the specific period when MicroTom has completed approximately 50% of its entire life cycle and is in the middle of the flowering time.

To date, only Kim et al., 2011 examined radiation sensitivity at two distinct vegetative and reproductive stages (i.e., 23 and 43 DAS, respectively). Unfortunately, these authors carried on their studies on *A. thaliana* treated with a 200 Gy dose (i.e., very different from our experimental setup oriented to a space scenario) and only evaluated leaf senescence in terms of photosynthetic ability and oxidative stress resistance.

In our experimental setup, plants intended to be irradiated at 45 DAS were seeded 15 days before plants that would have been irradiated at 30 DAS, in order to perform fluorimetric measurements simultaneously for both groups of plants and make them homogeneous, avoiding discrepancies due to the different contributions of natural light in the greenhouse. Due to this arrangement, plants experienced a synchronization that was evidenced by a delay recorded for the blossom period of the plants seeded 15 days before (plants to be irradiated at 45 DAS). This delay of the blossom period happened in non-irradiated internal controls for each genotype and, therefore could not be considered as an effect of irradiation. The synchronization of the flowering time can be ascribed to the role of volatiles organic compounds (VOCs) involved in plant communication (Bouwmeester et al., 2019). For this reason, measurements were accordingly extended.

As discussed for offspring of irradiated seeds and plants irradiated at 30 DAS, also treatments supplied to plants at 45 DAS resulted in different responses in function of genotype. Only wild type receiving the 5 Gy dose exhibited an increase in the number of leaves. This negative effect was compensated by an increased number of fruits and seeds. Unfortunately, a negative effect was noticed fruits-related parameters of AN4-P₂ plants, confirming that both life stages (30 and 45 DAS) are crucial periods for the correct plant development and that the responses to IR are somehow similar between these two stages. Also in this case, the irradiation at 30 Gy determined an overall significant negative effect, starting from 14-21 DAI (59-66 DAS) on the number of flowers, demonstrating that also at this developmental stage, between 5 and 30 Gy there is a limiting dose to complete the entire life cycle from seed-to-seed.

Fluorescence indices were differently influenced in dependence of irradiation dose supplied to the different genotypes in plants treated at 45 DAS. As somehow expected due to synchronization of the two experimental groups of plants, also the FLAV index, being related to secondary metabolism, overlapped in timing. This was made visible by the coincidence of the inversion point of the FLAV index of wild type plants with that of plants irradiated at 30 DAS (7-14 DAI). By contrast, the synchronization seemed to not affect the ANTH index, being the pigment either synthesized constitutively in the AN4 plants or not accumulated by wild type.

At the end of the detection period, differently from plants irradiated at 30 DAS, the FLAV index increased in irradiated wild type plants independently from the dose and in AN4-P₂ plants treated

with the 0.5 Gy dose. Along the detection period, it was possible to detect differences between responses of plants irradiated either at 30 DAS or at 45 DAS. While AN4-M plants responded with a clear increase in the FLAV index compared to untreated and the lower doses when irradiated at 30 DAS, the same plants did not respond at all when irradiated at 45 DAS. The same difference between 30 and 45 DAS was detectable for the ANTH values, as well. Even though less pronounced, the same ‘lack of response’ was noticed for SFR and NBI as if these were age-related responses to the irradiation stress. Age-related resistance to abiotic stress is achieved through changes in hormone-mediated responses and utilization of chemical defense mechanisms (Rankenberg et al., 2021). Nevertheless, it remains unclear how and which stress signals interact with and regulate established developmental regulators to modulate developmental progression and stress response capacity (e.g., stress escape, senescence, or photoprotection).

Although with some differences, we detected again the bi-phasic response described for plants irradiated at 30 DAS. In particular, this response resulted weaker if compared with that found in plants irradiated at 30 DAS (i.e., clearly visible only on the SFR index of AN4-M plants). Conversely, AN4-M showed a first-phase response (between 3 and 24 hours after the irradiation) with a decrease of the index and a second-phase response with a subsequent increase of it. By contrast, we were not able to detect a bi-phasic response trend for the NBI index, which showed a decrease in AN4 plants irradiated with the 30 Gy dose (from 28 to 42 DAI). As seen for offspring of irradiated seeds and plants irradiated at 30 DAS, irradiation did not cause changes in photosynthesis performances.

Summarizing, the response of plants irradiated at 30 and 45 DAS were more similar each other, with the 30 DAS that showed more pronounced responses, especially for fluorescence-based indices. These data demonstrated that irradiation at different life stages (e.g., seeds, plants at 30 DAS and 45 DAS) can result in different morphological and physiological plant responses.

6. CONCLUSIONS

During 2021 the NASA rover Perseverance and the Chinese Rover Zhurong have landed on Mars, while the Parker Solar Probe and the Juno Probe have gathered suitable data from Venus and Jupiter, respectively. In addition, China completed the establishment of the new orbital space station ‘Tiangong’ intended to house human beings. The year 2021 inaugurated the so-called "space tourism", with Virgin Galactic, Blue Origin and SpaceX leading their first manned launches, opening up for short trips just beyond the atmosphere. Meanwhile, SpaceX has continued to supply the ISS and transport the astronauts to the station, as well as testing its spaceships to reach Mars in the near future. The year ended with another great success: the launch of the James Webb Space Telescope, the largest and most powerful space telescope ever created. In 2022 NASA, with the Artemis program, is going to test the Space Launch System (SLS) carrying the Orion capsule that will be used in deep space manned mission. During this year, USA, Europe, India, Russia and Japan are going to launch ten space missions with the aim to return on the Moon. All these events demonstrate a great commitment in missions pointing at Moon, Mars and outer space in general.

The survival of human beings in outer space will depend on the knowledge acquired in different fields, together with the capacity to reproduce an environment able to simulate the symbiotic relationships between higher plants, animals and microorganisms. In these environments, higher plants will produce healthy food, and will possibly serve as bioreactors for the *in situ* production of medicines such as vaccines, antibodies and therapeutic molecules through plant molecular farming applications. Plants are expected to provide psychological support, as well.

Considering that the space environment is hostile to life for both humans and plants, it will be necessary to design biofortified plant ideotypes able to counteract space adverse conditions to protect humans (as the basis of anti-oxidant space food) and themselves from oxidative damage. This PhD work represented a preliminary study in the development of tomato plant ideotypes with future agrospace applications. MicroTom is considered a valuable candidate for agrospace applications. We found that the ectopic expression of the *Petunia hybrida* R2R3-MYB transcription factor PhAN4 in a MicroTom hairy root culture model deeply influenced the phenylpropanoid pathway, restoring anthocyanin biosynthesis and accumulation in tomato cells. Interestingly, a transcriptome reprogramming, oriented to positive regulation of cell response to biotic, abiotic and redox stimuli, was found. AN4 HRCs showed significant capability to counteract ROS accumulation and protein misfolding upon high dose gamma irradiation. The results obtained in the HRCs model demonstrate their versatility as a handier tool to study

specialized metabolite pathways and opened our way to whole MicroTom plant engineering. Transgenic lines harbouring one copy of *PhAN4* on a single locus were characterized. The ectopic expression of *PhAN4* in tomato plants resulted in the accumulation of anthocyanins in fruits and in an increased antioxidant scavenging capacity compared to wild type. In addition, engineered fruits evaluated upon gamma irradiation showed a greater capacity to counteract the generation of ROS and to preserve plant protein folding compared to wild type. These results could support the possible use of engineered MicroTom plants as an enriched food to counteract the risk of oxidative damage caused by the chronic exposure to cosmic radiation during long-term missions.

Exposure of engineered MicroTom to ionizing radiation, at doses that plants would possibly experience during different space mission scenarios, was reproduced on a lab scale using ground-based facilities. Engineered tomato seeds and plants at two developmental stages demonstrated that the response to gamma rays depends on the irradiation dose and developmental stage considered. With this work we tried to tackle the lack of knowledge regarding the effects of gamma rays on fully developed (tomato) plants and on offspring of irradiated plants. Plant responses to gamma rays were assessed using a double approach: measuring morphometric parameters we evaluated phenotypic changes while the use of fluorescent-based indices allowed us to indirectly monitor metabolic processes in a non-destructive manner.

Data obtained from irradiated dry seeds confirmed that, at this phenological stage, seeds are more resistant than fully developed plants to ionizing radiation, due to their structure. Data obtained from irradiation at 30 and 45 DAS demonstrated that between 5 and 30 Gy a limiting dose exists beyond which plants can't handle complete life cycle through the production of fruits and viable seeds. On the contrary, plants can tackle gamma ray doses until 5 Gy through adjustments at the physiological level. Indeed, the overall results suggest that low doses of ionizing radiation delivered at both seed stage and fully developed plants do not impair plants growth but, in some cases, can lead to beneficial traits (e.g., smaller size or increased number of seeds). Interestingly, fluorescence-based indices allowed us to detect a bi-phasic response to IR in plants irradiated at 30 DAS, while an age-related resistance to stress tolerance is possibly involved in the 'lack of response' showed by plants irradiated at 45 DAS.

We found that parental irradiation had consequences on offspring, but mostly affected parameters that were different from those influenced in parents, demonstrating that they do not depend on inheritable mutations of genes, and may be ascribed to long-term changes via epigenetic modifications and altered frequency of transitional recombination.

Lastly, despite previous studies on MicroTom showed that the

We confirmed in offspring of irradiated seeds that perturbations to the photosynthetic apparatus do not affect its efficiency and, for the first time, we demonstrated that this can be replicated by irradiated fully developed plants and their offspring.

AN4-M turned out to be the genotype with the best performance in tackling the stress of gamma ray exposure under different aspects, probably due to the most favourable genomic asset (homozygosity) determining the most adequate/balanced commitment of metabolism in both bioaccumulation of anthocyanins and stress response. These results may hopefully lead to the possibility of using this engineered MicroTom for the study of human consumption of space ideotypes on board of spacecraft or stationary bases.

In general, the results obtained in this work, may be beneficial in the understanding of some of the effect of space harsh conditions on plant growth, and lead to the exploitation of new breeding efforts to optimize plant performances to develop appropriate ideotype for future long-term space exploration.

APPENDIX

Appendix I. List of differentially expressed genes (DEGs) obtained from the RNA-seq analysis conducted on AN4 HRCs.

Up regulated DEGs		
Gene ID	Log ₂ FC	Gene description
Solyc07g04369 0.2.1	2,800732902	3-hydroxyisobutyryl-CoA_hydrolase-like_protein_(AHRD_V3.3_***_A0A072V5U1_MEDTR)
Solyc01g10539 0.3.1	5,100208627	Phospho-2-dehydro-3-deoxyheptonate_aldolase_(AHRD_V3.3_***_K4B2A9_SOLLC)
Solyc11g01724 0.2.1	3,78371117	chorismate_mutase
Solyc10g07517 0.2.1	4,196607745	Aspartate_aminotransferase_(AHRD_V3.3_***_A0A067JJ16_JATCU)
Solyc04g08113 0.1.1	3,67069064	LOW_QUALITY:glyoxal_oxidase-related_protein_(AHRD_V3.3_***_AT1G19900.1)
Solyc06g07376 0.3.1	4,071580028	Beta-glucosidase_(AHRD_V3.3_***_A0A0B4PJM3_SOYBN)
Solyc01g10665 0.3.1	3,003680242	Xyloglucan_endotransglucosylase/hydrolase_(AHRD_V3.3_***_K4B2N2_SOLLC)
Solyc03g09339 0.3.1	2,390710296	beta_expansin_precursor_2
Solyc05g05224 5.1.1	2,186779111	Expansin_(AHRD_V3.3_***_G4XZW3_9GENT)
Solyc05g05225 0.3.1	2,197851065	Expansin_(AHRD_V3.3_***_G4XZW3_9GENT)
Solyc08g07791 0.3.1	3,227703527	Expansin-like_protein_(AHRD_V3.3_***_W9S0F1_9ROSA)
Solyc09g01086 0.3.1	2,630126243	expansin_precursor_4
Solyc05g05436 0.3.1	6,837513657	Pectinesterase_(AHRD_V3.3_***_K4C2B4_SOLLC)
Solyc12g08865 0.2.1	3,003689155	cyclinD3_2
Solyc10g08393 0.2.1	2,363143147	Peptidyl-prolyl_cis-trans_isomerase_(AHRD_V3.3_***_K4D3H9_SOLLC)
Solyc12g03665 7.1.1	6,814156849	Tubulin_beta_chain_(AHRD_V3.3_***_A0A0D3BFN8_BRAOL)
Solyc03g11639 0.3.1	2,962695707	Late_embryogenesis_abundant_protein_(AHRD_V3.3_***_E1AZA3_SOLLC)
Solyc10g08069 0.2.1	2,304387313	Patatin_(AHRD_V3.3_***_K4D2V9_SOLLC)
Solyc01g06685 0.3.1	3,931286741	WAT1-related_protein_(AHRD_V3.3_***_K4AWT5_SOLLC)
Solyc03g00588 0.3.1	3,738441385	Bidirectional_sugar_transporter_SWEET_(AHRD_V3.3_***_K4BE11_SOLLC)
Solyc03g12062 0.3.1	2,545504462	Homeobox_leucine_zipper_protein_(AHRD_V3.3_***_G7IUR3_MEDTR)
Solyc06g07564 0.2.1	2,250469408	Tetratricopeptide_repeat_(TPR)-like_superfamily_protein_(AHRD_V3.3_***_AT4G20900.2)
Solyc10g08345 0.2.1	9,977092784	NAC_domain_protein_(AHRD_V3.3_***_A0JBY8_9ASPA)
Solyc12g00668 0.2.1	3,648537571	Early_nodulin_93_protein_(AHRD_V3.3_***_A3FGC9_9ROSI)

Solyc12g05606 0.2.1	3,310406275	Myosin_heavy_chain-related_family_protein_(AHRD_V3.3_***_B9GX71_POPTR)
Solyc06g06819 0.2.1	2,028019916	Ubiquitin_system_component_Cue_protein_putative_(AHRD_V3.3_*- *_A0A061GFQ5_THECC)
Solyc02g03869 0.1.1	3,637995718	Histone_H2B_(AHRD_V3.3_***_K4B5W3_SOLLC)
Solyc06g00539 0.1.1	7,505475064	Histone_H2B_(AHRD_V3.3_***_K4C323_SOLLC)
Solyc11g06643 0.1.1	2,481236637	histone_H2B
Solyc03g11907 0.2.1	5,597311433	LOW_QUALITY:Flap_endonuclease_1_(AHRD_V3.3_***_A0A0K9P479_ZOSMR)
Solyc12g03522 5.1.1	9,263765052	BED_zinc_finger,hAT_family_dimerization_domain_putative_(AHRD_V3.3_***_A 0A061FD65_THECC)
Solyc09g00884 0.3.1	2,154753327	Pyruvate_kinase_family_protein_(AHRD_V3.3_***_AT3G52990.1)
Solyc11g01242 0.2.1	3,228244777	Auxin_induced-like_protein_(AHRD_V3.3_***_A0A0K9NX81_ZOSMR)
Solyc01g06841 0.3.1	3,202548809	SIPIN5
Solyc08g06192 0.2.1	3,818406878	cytokinin_oxidase/dehydrogenase_3
Solyc03g12053 0.3.1	2,054979455	basic_helix-loop-helix_(bHLH)_DNA-binding_superfamily_protein_(AHRD_V3.3_*- *_AT4G29100.1)
Solyc07g02665 0.3.1	2,405513509	1-aminocyclopropane-1-carboxylate_oxidase_5
Solyc02g08387 0.3.1	3,217776859	Gibberellin-regulated_protein_2_putative_(AHRD_V3.3_***_B9SLF4_RICCO)
Solyc02g08388 0.3.1	2,409311445	Gibberellin-regulated_protein_2_putative_(AHRD_V3.3_***_B9SLF4_RICCO)
Solyc03g11606 7.1.1	3,831581265	Gibberellin-regulated_protein_(AHRD_V3.3_***_B4UW77_ARAHY)
Solyc11g01121 0.2.1	2,189943987	Gibberellin-regulated_family_protein_(AHRD_V3.3_***_AT1G74670.1)
Solyc01g00658 0.3.1	3,193807732	2-oxoglutarate-dependent_dioxygenase- related_family_protein_(AHRD_V3.3_***_B9GL08_POPTR)
Solyc01g05803 0.2.1	8,08401203	Gibberellin_2-oxidase_(AHRD_V3.3_***_A4GVL5_SOLLC)
Solyc06g06893 0.2.1	3,252699554	Jasmonate_ZIM_domain_protein_g_(AHRD_V3.3_***_I3WTA5_NICAT)
Solyc12g05694 0.2.1	2,583131083	Acetyl-CoA_carboxylase_family_protein_(AHRD_V3.3_***_B9GUK0_POPTR)
Solyc02g07702 0.3.1	3,462467053	Phospholipase_A1_(AHRD_V3.3_***_A5YW95_CAPAN)
Solyc03g08337 0.3.1	8,370864195	Lipase_class_3_family_protein_(AHRD_V3.3_***_B9GXJ5_POPTR)
Solyc02g08334 0.3.1	2,409508625	Phospholipase_D_(AHRD_V3.3_*-*_K4BAK2_SOLLC)
Solyc10g07911 0.2.1	5,346090811	1-acyl-sn-glycerol-3- phosphate_acyltransferase_(AHRD_V3.3_***_Q2Z1Y8_PRUMU)
Solyc07g05269 5.1.1	4,565883395	Beta-amylase_(AHRD_V3.3_***_K4CFG8_SOLLC)
Solyc09g09243 0.3.1	2,525751915	Selenium_binding_family_protein_(AHRD_V3.3_***_B9GFI5_POPTR)
Solyc01g00846 0.3.1	2,236566603	Ferredoxin_(AHRD_V3.3_***_Q70SW4_DIGLA)

Solyc09g00961 0.2.1	7,010471146	Purple_acid_phosphatase_(AHRD_V3.3_***_K4CQZ0_SOLLC)
Solyc12g06225 0.2.1	2,426147815	like_SEX4_1_(AHRD_V3.3_***_AT3G01510.1)
Solyc01g05998 0.3.1	3,453518422	Beta-1,3-glucanase_(AHRD_V3.3_***_G9G7S0_HEVBR)
Solyc01g05996 5.1.1	3,476158885	Beta-1,3-glucanase_(AHRD_V3.3_***_G9G7S0_HEVBR)
Solyc02g07045 0.2.1	3,085855552	Glucan_endo-1,3-beta-glucosidase_putative_(AHRD_V3.3_***_B9S109_RICCO)
Solyc01g09628 0.2.1	2,145366925	Cytochrome_P450_(AHRD_V3.3_***_F4YF83_9APIA)
Solyc02g06523 0.3.1	3,170387722	Cytochrome_P450_(AHRD_V3.3_***_Q8H0I6_PETHY)
Solyc02g08588 0.2.1	3,005308809	Cytochrome_P450_putative_(AHRD_V3.3_***_B9RSK4_RICCO)
Solyc07g06447 0.3.1	6,968906136	Cytochrome_P450_(AHRD_V3.3_***_A0A061FD55_THECC)
Solyc09g05924 0.3.1	3,07006597	Cytochrome_P450_putative_(AHRD_V3.3_***_B9SYM4_RICCO)
Solyc10g08155 0.2.1	2,902706497	Cytochrome_P450_putative_(AHRD_V3.3_***_B9SYM5_RICCO)
Solyc12g04498 3.1.1	2,732915259	Cytochrome_P450_family_protein_(AHRD_V3.3_***_B9HFV6_POPTR)
Solyc12g09684 0.2.1	5,313066603	Tyramine_N-feruloyltransferase_4/11_putative_(AHRD_V3.3_***_B9T0B8_RICCO)
Solyc12g04955 0.2.1	3,50145272	GDSL_esterase/lipase_5_(AHRD_V3.3_***_GLIP5_ARATH)
Solyc10g04524 0.2.1	2,022207191	Beta-glucosidase_(AHRD_V3.3_***_L0ASF2_POPTO)
Solyc12g04064 0.2.1	3,39047071	Beta-glucosidase_putative_(AHRD_V3.3_***_B9T4F7_RICCO)
Solyc02g08134 0.3.1	10,53476424	Glutathione_S-transferase_(AHRD_V3.3_***_E1AWJ7_CAPAN)
Solyc01g10868 0.3.1	5,874157371	Methyl_esterase_(AHRD_V3.3_***_A0A072UEL6_MEDTR)
Solyc06g06260 0.3.1	2,889632023	Glucose-methanol-choline_(GMC)_oxidoreductase_family_protein_(AHRD_V3.3_***_AT1G72970.1)
Solyc09g09170 0.3.1	8,727396556	NADP-dependent_alkenal_double_bond_reductase_(AHRD_V3.3_***_I3SE71_MEDTR)
Solyc02g06251 0.3.1	2,392338587	Peroxidase_(AHRD_V3.3_***_K4B6D9_SOLLC)
Solyc02g07730 0.2.1	7,236518572	Peroxidase_(AHRD_V3.3_***_K4B8V7_SOLLC)
Solyc04g08076 0.3.1	2,42913063	Peroxidase_(AHRD_V3.3_***_K4BVF5_SOLLC)
Solyc01g10440 0.3.1	2,103323448	Basic_blue_protein_(AHRD_V3.3_***_A0A151R520_CAJCA)
Solyc09g07581 0.3.1	2,241561781	Early_nodulin-like_protein_(AHRD_V3.3_***_A0A0K9P6C2_ZOSMR)
Solyc11g06235 0.2.1	3,082652842	Blue_copper_protein_(AHRD_V3.3_***_A0A199UNZ4_ANACO)
Solyc01g08160 0.3.1	2,051661627	Non-specific_lipid-transfer_protein_(AHRD_V3.3_***_K4AY52_SOLLC)
Solyc03g00520 0.3.1	4,493815367	Lipid_transfer_protein_(AHRD_V3.3_***_G7K520_MEDTR)

Solyc03g11921 0.1.1	2,351957294	Lipid_transfer_protein_(AHRD_V3.3_***_S4TID2_GOSHI)
Solyc08g00596 0.2.1	2,771272559	Bifunctional_inhibitor/lipid-transfer_protein/seed_storage_2S_albumin_superfamily_protein_(AHRD_V3.3_***_AT2G45180.1)
Solyc02g06297 5.1.1	7,081891304	Glycosyltransferase_(AHRD_V3.3_***_K4B6I8_SOLLC)
Solyc05g01302 0.1.1	7,116641536	Xyloglucan_alpha-1,6-xylosyltransferase_(AHRD_V3.3_***_X5MPI5_PINTA)
Solyc09g05917 0.2.1	8,326262972	Glycosyltransferase_(AHRD_V3.3_***_M1C989_SOLTU)
Solyc10g08344 0.1.1	9,972475187	Glycosyltransferase_(AHRD_V3.3_***_K4D3D1_SOLLC)
Solyc12g09858 0.1.1	3,409153716	Glycosyltransferase_(AHRD_V3.3_***_K4DHN1_SOLLC)
Solyc12g09859 0.2.1	16,55105848	Glycosyltransferase_(AHRD_V3.3_***_K4DHN2_SOLLC)
Solyc08g04427 0.3.1	2,640975765	Glutamate_synthase_putative_(AHRD_V3.3_***_B9RII5_RICCO)
Solyc01g06791 0.3.1	5,061121609	Unknown_protein_(AHRD_V3.3_)
Solyc00g07210 0.3.1	2,168210786	Retrovirus-related_Pol_polyprotein_from_transposon_TNT_1-94_(AHRD_V3.3_***_A0A151S5P0_CAJCA)
Solyc01g10710 0.3.1	2,71199305	LOW_QUALITY:beta-1,4-xylosidase_(AHRD_V3.3_***_AT3G19615.1)
Solyc03g03189 0.3.1	2,9771873	Oxidative_stress_3_putative_isoform_2_(AHRD_V3.3_***_A0A061GIQ6_THECC)
Solyc03g09646 0.3.1	2,302142017	wound/stress_protein_AY568721
Solyc03g12060 0.3.1	5,539264566	Plant_cadmium_resistance_2_(AHRD_V3.3_***_A0A0K9NVT8_ZOSMR)
Solyc06g07399 0.2.1	3,819977516	Gb:AAF02129.1_putative_(AHRD_V3.3_***_A0A061GVZ4_THECC)
Solyc07g00742 0.3.1	3,07050649	F-box/LRR-repeat_protein_putative_(AHRD_V3.3_***_B9RVD1_RICCO)
Solyc07g04370 0.2.1	2,800732902	Acylsugar_acyltransferase_3_(AHRD_V3.3_***_ASAT3_SOLLC)
Solyc07g06492 0.3.1	2,582056411	Upstream_activation_factor_subunit_spp27_(AHRD_V3.3_***_A0A0B2NVJ3_GLYSO)
Solyc09g00707 0.1.1	3,118825075	Succinate_dehydrogenase_assembly_factor_1_mitochondrial_(AHRD_V3.3_***_A0A0B0PRI9_GOSAR)
Solyc09g01020 0.3.1	2,248015523	CASP-like_protein_(AHRD_V3.3_***_K4CR48_SOLLC)
Solyc09g07455 0.3.1	3,385659768	CASP-like_protein_(AHRD_V3.3_***_M1ASY5_SOLTU)
Solyc10g08325 0.2.1	2,085006445	CASP-like_protein_(AHRD_V3.3_***_K4D3B2_SOLLC)
Solyc10g08341 0.1.1	3,538039347	LOW_QUALITY:Peptidoglycan-binding_LysM_domain_protein_(AHRD_V3.3_***_A0A072VJR4_MEDTR)
Solyc11g00773 0.2.1	2,195159924	Bark_storage_protein_A_(AHRD_V3.3_***_W9SCI4_9ROSA)
Solyc12g04253 0.2.1	5,306033926	phosphatidylinositol-glycan_biosynthesis_class_F-like_protein_(AHRD_V3.3_***_AT1G16040.1)
Solyc12g08817 0.2.1	13,21347105	HXXXD-type_acyl-transferase_family_protein_putative_(AHRD_V3.3_***_A0A061E8Z9_THECC)

Solyc11g06484 0.1.1	7,476848358	LOW_QUALITY:Calcium-dependent_lipid-binding_(CaLB_domain)_family_protein_(AHRD_V3.3_***_G7K355_MEDTR)
Solyc02g08190 0.3.1	7,276987126	Pentatricopeptide_repeat-containing_protein_(AHRD_V3.3_***_A0A103YLQ2_CYNCS)
Solyc07g05522 0.2.1	3,250974432	Pentatricopeptide_repeat-containing_protein_putative_(AHRD_V3.3_***_B9T3G5_RICCO)
Solyc01g00621 0.3.1	3,706313194	Non-structural_maintenance_of_chromosomes_element_1_(AHRD_V3.3_***_A0A118K055_CYNCS)
Solyc01g05828 0.3.1	2,161706586	Transmembrane_93_(AHRD_V3.3_***_A0A0B0PFK3_GOSAR)
Solyc01g05873 3.1.1	2,112619964	embryo_sac_development_arrest_7_(AHRD_V3.3_--*_AT3G56990.2)
Solyc01g06690 0.3.1	2,284146072	cationic_amino_acid_transporter_9_(AHRD_V3.3_--*_AT1G05940.2)
Solyc01g06842 0.3.1	4,0737032	Trichome_birefringence-like_protein_(AHRD_V3.3_***_G7IKB9_MEDTR)
Solyc01g07955 0.2.1	2,07479698	LOW_QUALITY:Multidrug_resistance_protein_ABC_transporter_family_protein_putative_(AHRD_V3.3_***_A0A061DZ20_THECC)
Solyc01g09145 5.1.1	4,239568747	piezo-type_mechanosensitive_ion_channel_component_(AHRD_V3.3_--*_AT2G48060.3)
Solyc01g10044 0.2.1	3,111283403	ATP-dependent_DNA_helicase_(AHRD_V3.3_--*_AT2G24100.1)
Solyc01g10444 4.1.1	7,319389661	Electron_transfer_flavoprotein-ubiquinone_oxidoreductase_mitochondrial_(AHRD_V3.3_--*_M4CKM4_BRARP)
Solyc01g10631 0.3.1	5,217654887	cysteine-rich/transmembrane_domain_A-like_protein_(AHRD_V3.3_--*_AT2G32210.1)
Solyc01g10842 0.2.1	3,168142964	LOW_QUALITY:mitogen-activated_protein_kinase_kinase_kinase_3_(AHRD_V3.3_--*_AT1G53570.7)
Solyc01g10925 0.2.1	2,704152775	LOW_QUALITY:DUF4228_domain_protein_(AHRD_V3.3_***_I3SPG4_MEDTR)
Solyc01g10943 0.3.1	3,492462618	PPPDE_putative_thiol_peptidase_family_protein
Solyc01g11111 0.3.1	2,892754206	Oxidoreductase/transition_metal_ion-binding_protein_(AHRD_V3.3_--*_A2Q3C9_MEDTR)
Solyc02g06298 0.2.1	7,04757722	Glycosyltransferase_(AHRD_V3.3_--*_K4B6I8_SOLLC)
Solyc02g06344 0.3.1	7,994888252	cysteine-rich/transmembrane_domain_A-like_protein_(AHRD_V3.3_--*_AT2G32210.1)
Solyc02g06812 5.1.1	2,011915527	Photosystem_II_CP43_reaction_center_protein_(AHRD_V3.3_--*_PSBC_ACUOB)
Solyc02g07246 0.1.1	6,988946177	LOW_QUALITY:Ycf20-like_protein_(AHRD_V3.3_***_A0A1D1YWC0_9ARAE)
Solyc02g07756 5.1.1	4,988020178	RING/U-box_superfamily_protein_(AHRD_V3.3_--*_AT1G78420.2)
Solyc02g07876 0.1.1	3,401185983	LOW_QUALITY:BEST_plant_protein_match_is:(TAIR:plant.1)_protein_putative_(AHRD_V3.3_***_G7INX9_MEDTR)
Solyc02g07901 0.3.1	2,242727491	Cotton_fiber_expressed_protein_(AHRD_V3.3_***_A0A109Y892_GOSBA)
Solyc02g08946 0.1.1	2,109762273	Glutamine_dumper_2_putative_(AHRD_V3.3_***_A0A061ENI6_THECC)
Solyc02g08948 0.1.1	7,267174291	Glutamine_dumper_2_putative_(AHRD_V3.3_***_A0A061ENI6_THECC)
Solyc02g08966 0.2.1	2,24841426	Titin_(AHRD_V3.3_***_A0A0B0NSM2_GOSAR)

Solyc02g09012 0.1.1	2,475963255	LOW_QUALITY:Inositol_1,4,5-trisphosphate_receptor-interacting_protein-like_2_(AHRD_V3.3_**_A0A1D1XL30_9ARAE)
Solyc02g09228 5.1.1	2,588076354	aminophospholipid_ATPase_3_(AHRD_V3.3_--*_AT1G59820.1)
Solyc03g00684 0.3.1	5,084345876	LOW_protein: M-phase_inducer_phosphatase-like_protein_(AHRD_V3.3_***_AT5G62170.1)
Solyc03g06270 0.3.1	2,820748139	ARM_repeat_superfamily_protein_(AHRD_V3.3_***_A0A061F2F3_THECC)
Solyc03g06271 0.3.1	2,785746584	NDH-dependent_cyclic_electron_flow_5_(AHRD_V3.3_***_A0A0F7CYY3_9ROSI)
Solyc03g09575 3.1.1	8,231628962	Glycerol-3-phosphate_transporter_(AHRD_V3.3_**_A0A0A0KL51_CUCSA)
Solyc03g09581 0.3.1	6,923791313	Trichome_birefringence-like_protein_(AHRD_V3.3_***_G7K771_MEDTR)
Solyc03g09641 0.1.1	3,525892863	LOW_QUALITY:ARID/BRIGHT_DNA-binding_, ELM2_domain_and_myb-like_DNA-binding_domain-containing_protein_(AHRD_V3.3_--*_AT4G11400.2)
Solyc03g02010 0.2.1	3,600252339	LOW_QUALITY:Protein_Ycf2_(AHRD_V3.3_--*_YCF2_VITVI)
Solyc03g02008 0.3.1	2,017930333	Pin-II_type_proteinase_inhibitor_69_(AHRD_V3.3_***_A0A097J9C9_CAPAN)
Solyc03g02003 0.3.1	10,72904412	Pin-II_type_proteinase_inhibitor_69_(AHRD_V3.3_***_A0A097J9C9_CAPAN)
Solyc03g01987 7.1.1	2,041717343	TSL-kinase_interacting_protein_1_(AHRD_V3.3_--*_AT2G36960.3)
Solyc03g11089 0.1.1	4,014301881	LOW_QUALITY:glucuronoxylan_4-O-methyltransferase-like_protein_(DUF579)_(AHRD_V3.3_***_AT4G24910.1)
Solyc03g11098 7.1.1	2,901156771	Transducin/WD40_repeat-like_superfamily_protein_(AHRD_V3.3_--*_AT4G11270.2)
Solyc03g11470 5.1.1	3,172062279	DELLA_protein_(AHRD_V3.3_--*_Q0HA73_MALDO)
Solyc03g11680 0.3.1	2,323661939	glutamate_receptor_2.5_(AHRD_V3.3_--*_AT5G11210.9)
Solyc04g01149 5.1.1	4,789298939	LOW_QUALITY:transmembrane_protein_(AHRD_V3.3_***_AT4G26055.1)
Solyc04g01173 5.1.1	3,472594231	methyl-CPG-binding_domain_7_(AHRD_V3.3_--*_AT5G59800.1)
Solyc04g01428 0.1.1	6,968977025	LOW_QUALITY:hydroxyproline-rich_glycoprotein_family_protein_(AHRD_V3.3_***_AT5G09520.1)
Solyc04g01452 0.1.1	3,753496935	LOW_QUALITY:Transmembrane_protein_45B_(AHRD_V3.3_***_A0A151U0K4_CAJCA)
Solyc04g01607 0.2.1	4,773576668	LOW_QUALITY:MADS-box_transcription_factor_(AHRD_V3.3_***_A0A023JBP0_9ROSI)
Solyc04g05810 0.3.1	2,215238966	Metallothionein-like_protein_type_2_(AHRD_V3.3_***_MT2X_SOLLC)
Solyc04g06320 0.1.1	6,89899096	LOW_QUALITY:PH01B035L11.21_protein_(AHRD_V3.3_--*_L0P1Q5_9POAL)
Solyc04g07160 0.3.1	2,110881509	Absciscic_acid_stress_ripening_5
Solyc04g07723 0.1.1	2,69542807	Indole-3-acetaldehyde_oxidase_(AHRD_V3.3_--*_ALDO1_MAIZE)
Solyc05g00775 0.3.1	7,118706464	Cysteine-rich_receptor-like_protein_kinase_40_(AHRD_V3.3_--*_CRK40_ARATH)
Solyc05g00815 0.3.1	5,528269385	Metalloprotease_inhibitor_(AHRD_V3.3_***_O24373_SOLTU)
Solyc05g05072 0.3.1	2,234210231	Preprotein_translocase_subunit_SECE1_(AHRD_V3.3_***_A0A1D1Z4R7_9ARAE)

Solyc05g05174 0.3.1	3,353238915	Prosystemin_(AHRD_V3.3_--*_O48857_SOLNI)
Solyc05g05288 0.3.1	3,205503262	Plant/T7H20-70_protein_(AHRD_V3.3_***_A0A072U1L2_MEDTR)
Solyc05g05443 5.1.1	4,008896439	DNA_replication_complex_GINS_protein_PSF2_(AHRD_V3.3_--*_M1C604_SOLTU)
Solyc06g00530 0.3.1	3,885556754	TOX_high_mobility_group_box_family_member_4-A_putative_isoform_1_(AHRD_V3.3_***_A0A061H046_THECC)
Solyc06g05061 0.2.1	6,890275607	DCD_(Development_and_Cell_Death)_domain_protein_(AHRD_V3.3_--*_AT3G11000.1)
Solyc06g05087 0.3.1	5,322583112	Hypoxia-responsive_family_protein_(AHRD_V3.3_***_A0A061FYY0_THECC)
Solyc06g05380 5.1.1	3,984777815	cytochrome_P450_family_71_subfamily_B_polypeptide_30_pseudogene_(AHRD_V3.3_--*_AT3G53290.1)
Solyc06g06057 0.3.1	9,240692447	Dihydrofolate_reductase_(AHRD_V3.3_***_A0A0B0NHA3_GOSAR)
Solyc06g06279 5.1.1	8,484778499	LOW_QUALITY:DUF3511_domain_protein_(AHRD_V3.3_--*_G7LI91_MEDTR)
Solyc06g06798 0.3.1	2,631847769	Late_embryogenesis_abundant_protein_(AHRD_V3.3_--*_Q2QKE8_CATRO)
Solyc06g07426 0.3.1	6,932251026	pollen_proteins_Ole_e_I-like
Solyc06g08232 0.3.1	7,055273571	Pentatricopeptide_repeat-containing_protein_At5g12100_mitochondrial_(AHRD_V3.3_***_A0A1D1Z416_9_ARAE)
Solyc06g08356 0.3.1	8,80950271	transducin_family_protein/_WD-40_repeat_family_protein_(AHRD_V3.3_--*_AT5G08560.3)
Solyc06g08417 0.3.1	2,72848035	low-molecular-weight_cysteine-rich_56_(AHRD_V3.3_--*_AT3G20993.1)
Solyc06g08418 0.3.1	2,59353121	Plant_thionin_family_protein_(AHRD_V3.3_--*_AT2G20619.1)
Solyc07g00602 0.3.1	2,308229872	ADIPOR-like_receptor_(AHRD_V3.3_***_W9QY38_9ROSA)
Solyc07g00775 0.3.1	4,773645757	Defensin_protein_(AHRD_V3.3_***_B1N678_SOLLC)
Solyc07g00801 5.1.1	2,721757997	Surfeit_locus_protein_5_subunit_22_of_Mediator_complex_(AHRD_V3.3_--*_AT1G16430.1)
Solyc07g05472 0.2.1	4,778537681	Type_II_proteinase_inhibitor_family_protein_(AHRD_V3.3_--*_B6SS5_MAIZE)
Solyc07g05481 0.1.1	3,501988403	LOW_QUALITY:glutamate-1-semialdehyde_2,1-aminomutase_2_(AHRD_V3.3_--*_AT3G48730.1)
Solyc07g06189 0.1.1	7,073926929	LOW_QUALITY:Heat_stress_transcription_factor_B-4b_putative_(AHRD_V3.3_***_A0A061FAG8_THECC)
Solyc07g06317 0.1.1	2,042514372	Sodium/calcium_exchanger_family_protein_(AHRD_V3.3_--*_AT5G17850.2)
Solyc07g06469 0.1.1	2,077545086	LOW_QUALITY:50S_ribosomal_protein_L34_(AHRD_V3.3_***_AT1G29040.1)
Solyc08g04840 0.2.1	6,000221353	RNA_helicase_ATP-dependent_SK12/DOB1_protein_(AHRD_V3.3_--*_AT3G46960.3)
Solyc08g07570 5.1.1	4,258074963	thioredoxin_Y2_(AHRD_V3.3_--*_AT1G43560.1)
Solyc08g07657 0.3.1	3,859364213	Phosphatidylinositol-glycan_biosynthesis_class_X_protein_putative_(AHRD_V3.3_***_A0A061GDT5_T_HECC)

Solyc08g08147 0.3.1	2,05026296	3- isopropylmalate_dehydratase_large_subunit_(AHRD_V3.3_***_A0A0B0Q086_GOSAR)
Solyc09g05586 0.1.1	5,407044593	LOW_QUALITY:Plant_invertase/pectin_methylesterase_inhibitor_superfamily_protein_(AHRD_V3.3_--*_AT2G31432.2)
Solyc09g08281 0.3.1	4,784967005	Glycine-rich_protein_(AHRD_V3.3_--*_D2K2U0_TOBAC)
Solyc09g09777 0.3.1	2,907428152	Glycine-rich_protein_(AHRD_V3.3_***_D2K2T8_TOBAC)
Solyc10g00612 0.2.1	7,357873128	extracellular_ligand-gated_ion_channel_protein_(DUF3537)_(AHRD_V3.3_***_AT3G20300.1)
Solyc10g00655 0.2.1	6,146162869	Protein_DETOTOXIFICATION_(AHRD_V3.3_--*_A0A0D3HQG4_9ORYZ)
Solyc10g04976 0.1.1	3,991260947	Signal_anchor_putative_(AHRD_V3.3_--*_A0A072UJ16_MEDTR)
Solyc10g05098 0.1.1	5,843118026	PAB-dependent_poly(A)-specific_ribonuclease_subunit_PAN2_(AHRD_V3.3_--*_A0A1D1XPR6_9ARAE)
Solyc10g05099 0.1.1	5,934806808	Pyruvate_kinase_(AHRD_V3.3_--*_A9TZX1_PHYPA)
Solyc10g07640 0.2.1	2,650086714	Trichome_birefringence-like_protein_(AHRD_V3.3_***_G7JKP3_MEDTR)
Solyc10g07981 0.1.1	2,520052328	LOW_QUALITY:mediator_of_RNA_polymerase_II_transcription_subunit_(AHRD_V3.3_--*_AT2G10440.2)
Solyc10g08452 0.1.1	2,851754047	RNAse_THREE-like_protein_3_(AHRD_V3.3_--*_AT5G45150.1)
Solyc11g00713 0.2.1	3,002804801	Hippocampus_abundant_transcript-like_protein_1_(AHRD_V3.3_***_W9QGN7_9ROSA)
Solyc11g00741 0.2.1	2,784551956	Receptor-like_protein_kinase_(AHRD_V3.3_--*_A0A061EYK6_THECC)
Solyc11g00786 0.1.1	4,086002294	LOW_QUALITY:Cyclin-dependent_protein_kinase_inhibitor_Siamese_(AHRD_V3.3_***_M4M6N3_GOSAR)
Solyc11g01739 0.2.1	7,122197427	LOW_QUALITY:RNA-binding_KH_domain-containing_protein_(AHRD_V3.3_--*_AT5G46190.4)
Solyc11g02096 0.2.1	2,362943887	Proteinase_inhibitor_II_(AHRD_V3.3_--*_B3F0C1_TOBAC)
Solyc11g02127 0.1.1	2,94364162	Ycf1_(AHRD_V3.3_--*_V5NDV5_9SOLN)
Solyc11g06695 0.2.1	2,955987551	Stress_responsive_A/B_Barrel_Domain-containing_protein_(AHRD_V3.3_***_AT5G22580.1)
Solyc11g07135 0.1.1	2,037443817	aluminum_activated_malate_transporter_family_protein_(AHRD_V3.3_***_AT1G08440.1)
Solyc12g00580 0.2.1	7,517786731	Myb_transcription_factor_(AHRD_V3.3_***_A0A072UKM9_MEDTR)
Solyc12g00581 0.1.1	7,41352223	LOW_QUALITY:Exocyst_complex_component_SEC5_(AHRD_V3.3_--*_AT1G21170.2)
Solyc12g00998 0.2.1	2,46647714	UPF0133_protein_(AHRD_V3.3_***_A0A0B2RB99_GLYSO)
Solyc12g01955 0.2.1	4,891474458	Lipase_maturation_factor_2_(AHRD_V3.3_***_A0A1D1ZBR5_9ARAE)
Solyc12g03522 3.1.1	7,758549439	Cytochrome_b561_and_DOMON_domain-containing_protein_At5g35735_(AHRD_V3.3_--*_B561P_ARATH)
Solyc12g04254 0.1.1	6,798777388	Ephrin-A3_(AHRD_V3.3_***_A0A0B0PTB6_GOSAR)
Solyc12g04921 0.1.1	2,85205982	LOW_QUALITY:DUF3511_domain_protein_(AHRD_V3.3_***_G7IS34_MEDTR)

Solyc12g09438 0.2.1	3,412028086	Thioredoxin_superfamily_protein_(AHRD_V3.3_***_A0A061FEH2_THECC)
Solyc12g09703 5.1.1	3,392550115	Small_acidic_protein_1_(AHRD_V3.3_***_A0A061EN42_THECC)
Solyc12g09880 0.2.1	8,02720692	LOW_QUALITY:Extensin_(AHRD_V3.3_--*_Q9FSG0_NICSY)
Solyc12g09854 0.2.1	2,925715171	Apyrase_(AHRD_V3.3_***_APY_SOLTU)
Solyc06g05351 0.3.1	3,104995239	putrescine_N-methyltransferase
Solyc06g05352 0.3.1	3,437644609	spermidine_synthase_(AHRD_V3.3_***_AT1G23820.1)
Solyc04g03976 0.2.1	2,616335336	Protein_Ycf2_(AHRD_V3.3_*-*_YCF2_SOLLC)
Solyc05g04744 0.1.1	3,459747607	LOW_QUALITY:Ycf1_protein_(AHRD_V3.3_*-*_ATCG01130.1)
Solyc09g03183 0.1.1	2,245841698	Protein_Ycf2_(AHRD_V3.3_*-*_YCF2_TOBAC)
Solyc10g01899 0.2.1	2,638476183	Protein_Ycf2_(AHRD_V3.3_*-*_YCF2_SOLLC)
Solyc08g07698 0.3.1	7,474442632	Acetylornithine_deacetylase_(AHRD_V3.3_***_ARGE_ARATH)
Solyc10g08130 0.1.1	2,564715	Metacaspase_9_(AHRD_V3.3_***_M4Y069_CAPAN),Pfam:PF00656
Solyc01g11011 0.3.1	2,177353221	Cysteine_proteinase_(AHRD_V3.3_***_C0KIY3_SOLLC)
Solyc02g07671 0.3.1	2,307987898	Cysteine_protease_(AHRD_V3.3_***_Q7XYU8_ANTAD)
Solyc05g00531 0.2.1	3,378328312	NEDD8-specific_protease_1_(AHRD_V3.3_***_W9SIH8_9ROSA)
Solyc08g08063 0.3.1	2,33279266	Ethylene-responsive_proteinase_inhibitor_1_(AHRD_V3.3_***_IER1_SOLLC)
Solyc10g08609 0.2.1	2,247200225	Trypsin_inhibitor_1_(AHRD_V3.3_***_ITR1_NICSY)
Solyc01g09656 0.2.1	4,109312617	Subtilisin-like_protease_(AHRD_V3.3_***_A0A0B2PRM9_GLYSO)
Solyc03g00747 0.3.1	2,301809829	Ubiquitin-conjugating_enzyme_E2_(AHRD_V3.3_***_A0A0K9PK38_ZOSMR)
Solyc11g06519 0.2.1	3,538329343	Ubiquitin-conjugating_enzyme_(AHRD_V3.3_***_G7K9A0_MEDTR)
Solyc12g01050 0.2.1	5,358159809	RING/U-box_superfamily_protein_(AHRD_V3.3_*-*_AT3G47180.1)
Solyc12g01067 0.1.1	5,364030417	RING/U-box_superfamily_protein_(AHRD_V3.3_*-*_AT1G74990.1)
Solyc06g04311 3.1.1	2,927343539	Serine/threonine_protein_phosphatase_7_long_form_isogeny_(AHRD_V3.3_*-*_A0A151R878_CAJCA)
Solyc02g08079 0.3.1	3,317661852	Deoxyhypusine_synthase
Solyc01g01708 7.1.1	2,613921769	30S_ribosomal_protein_S18_chloroplastic_(AHRD_V3.3_***_RR18_TOBAC)
Solyc02g01181 5.1.1	4,268639642	30S_ribosomal_protein_S15_chloroplastic_(AHRD_V3.3_*-*_RR15_SOLLC)
Solyc10g08633 0.2.1	2,085696249	60S_ribosomal_protein_L23a_(AHRD_V3.3_***_RL23A_DAUCA)
Solyc11g07153 0.1.1	2,15078417	50S_ribosomal_protein_L7/L12_(AHRD_V3.3_***_A0A0B2SQD1_GLYSO)

Solyc01g00725 0.3.1	2,717506567	U3_small_nucleolar_ribonucleoprotein_protein_imp4_putative_(AHRD_V3.3_***_B9RGD5_RICCO)
Solyc02g07212 0.3.1	2,254143452	ER_lumen_protein-retaining_receptor_(AHRD_V3.3_***_K4B8I6_SOLLC)
Solyc10g04743 0.1.1	7,271740694	LOW_QUALITY:Ribulose_bisphosphate_carboxylase_large_chain_(AHRD_V3.3_**_Q6SSH3_9SPER)
Solyc02g02129 0.1.1	3,023930642	LOW_QUALITY:Photosystem_II_32_kDa_protein_(AHRD_V3.3_**_G4VY79_9MARC)
Solyc12g00614 0.2.1	4,364619823	Cab-5_gene_encoding_chlorophyll_a/b-binding_protein
Solyc08g08003 0.3.1	5,628389471	Glyoxylate_reductase_(AHRD_V3.3_***_A0A0B0NZ21_GOSAR)
Solyc04g07814 0.3.1	8,35041607	Cytochrome_B5_(AHRD_V3.3_***_Q9ZSP7_PETHY)
Solyc06g08344 0.3.1	2,241703061	Cytochrome_b5_(AHRD_V3.3_***_CYB5_TOBAC)
Solyc06g00942 0.3.1	2,472244447	Pentatricopeptide_repeat-containing_protein_(AHRD_V3.3_**_A0A0B2SEE4_GLYSO)
Solyc07g00657 0.3.1	4,221848981	Ribonuclease_(AHRD_V3.3_***_Q41722_ZINVI)
Solyc02g08591 0.3.1	3,378940313	LOB_domain-containing_protein_(AHRD_V3.3_***_A0A0K9PUI8_ZOSMR)
Solyc06g05383 0.3.1	3,38035307	auxin-regulated_IAA7
Solyc02g09144 0.2.1	2,129583909	basic_helix-loop-helix_(bHLH)_DNA-binding_superfamily_protein_(AHRD_V3.3_**_AT2G14760.1)
Solyc09g06510 0.2.1	14,18672027	bHLH_transcription_factor_150
Solyc12g08813 0.2.1	4,327213562	basic_helix-loop-helix_(bHLH)_DNA-binding_superfamily_protein_(AHRD_V3.3_***_AT5G10570.1)
Solyc01g09076 0.3.1	2,250443176	GATA_transcription_factor_(AHRD_V3.3_***_K4AZ17_SOLLC)
Solyc05g00631 0.2.1	2,855278996	Zinc_finger_protein_putative_(AHRD_V3.3_**_B9RSL7_RICCO)
Solyc10g07872 0.2.1	2,074664131	Myb_family_transcription_factor_family_protein_(AHRD_V3.3_***_B9HAD3_POPTR)
Solyc07g05484 0.3.1	3,341226163	R2R3MYB_transcription_factor_41
Solyc10g08390 0.2.1	3,542474125	R2R3MYB_transcription_factor_27
Solyc10g08629 0.2.1	8,651275639	R2R3MYB_transcription_factor_114
Solyc01g09564 0.2.1	4,928760848	Myb_family_transcription_factor_(AHRD_V3.3_***_A0A0K9P4S6_ZOSMR)
Solyc07g05249 0.3.1	7,173073383	Myb_transcription_factor_(AHRD_V3.3_**_A0A072UKM9_MEDTR)
Solyc04g07778 0.3.1	2,019567752	Transcription_factor_LIM_(AHRD_V3.3_***_V9LXM6_TOBAC)
Solyc10g05114 0.2.1	4,849515562	Enhanced_downy_mildew_2_putative_isoform_1_(AHRD_V3.3_***_A0A061DLT4_THECC)
Solyc01g00827 0.3.1	2,143534304	Prefoldin_subunit_5_(AHRD_V3.3_***_B6TVU2_MAIZE)
Solyc03g09400 0.3.1	4,107424627	Light_induced_like_protein_(AHRD_V3.3_***_C1E3F8_MICCC)
Solyc06g05462 0.3.1	2,223278371	Zinc_finger_transcription_factor_43

Solyc08g07706 0.3.1	2,541120261	Zinc_finger_LSD1-type_(AHRD_V3.3_***_A0A103XQX8_CYNCS)
Solyc08g08268 0.3.1	2,492456675	RING/U-box_superfamily_protein_(AHRD_V3.3_***_AT4G24015.1)
Solyc10g00797 0.2.1	6,961895177	WRKY_transcription_factor_77
Solyc10g08438 0.1.1	3,439413134	WRKY_transcription_factor_44
Solyc05g05343 0.3.1	3,426332544	DNA-directed_RNA_polymerase_subunit_(AHRD_V3.3_***_K4C223_SOLLC)
Solyc05g05265 0.2.1	5,184103812	LOW_QUALITY:HXXXD-type_acyl-transferase_family_protein_(AHRD_V3.3_*- *_V5PZN5_9CARY)
Solyc08g08004 0.3.1	8,861102345	Anthocyanidin_synthase_(AHRD_V3.3_***_A0A0A1E1Z9_9SOLA)
Solyc05g01032 0.3.1	4,308610241	Chalcone- flavonone_isomerase_family_protein_(AHRD_V3.3_***_K4BXX5_SOLLC)
Solyc05g05224 0.3.1	8,452278685	Chalcone- flavonone_isomerase_family_protein_(AHRD_V3.3_***_A0A0V0H4Z0_SOLCH)
Solyc05g05355 0.3.1	9,83030415	chalcone_synthase_2
Solyc09g09151 0.3.1	12,25514456	chalcone_synthase_1
Solyc02g08386 0.3.1	6,299607501	flavanone_3-dioxygenase
Solyc02g08502 0.3.1	10,09460014	dihydroflavonol_4-reductase
Solyc11g06658 0.2.1	16,35234434	flavonoid_3`%2C5`-hydroxylase
Solyc01g10588 0.4.1	3,469067651	Monoterpenoid_synthase_2
Solyc09g09247 0.2.1	5,695783056	sesquiterpene_synthase_14
Solyc06g05113 0.1.1	2,795062967	LOW_QUALITY:Transferase_family_protein_(AHRD_V3.3_***_B9HSL5_POPTR)
Solyc09g08266 0.3.1	15,05027118	Caffeoyl-CoA_O-methyltransferase_(AHRD_V3.3_***_Q2YHM9_PLAMJ)
Solyc11g01099 0.2.1	2,019664256	Alcohol_dehydrogenase_C-terminal_(AHRD_V3.3_***_A0A103YGD7_CYNCS)
Solyc10g00820 0.3.1	5,111196495	Tyrosine_aminotransferase_putative_(AHRD_V3.3_***_B9RVV0_RICCO)
Solyc01g05770 0.1.1	7,026827135	Calcium-dependent_protein_kinase_(AHRD_V3.3_*-*_H6UM40_TOBAC)
Solyc08g03659 0.1.1	5,413348568	Calcineurin_B_putative_(AHRD_V3.3_***_B9RCU6_RICCO)
Solyc12g00963 0.2.1	5,183567126	Calcium-binding_family_protein_(AHRD_V3.3_***_B9HA89_POPTR)
Solyc03g03177 0.3.1	7,427166287	ROP-interactive_CRIB_motif_protein_(AHRD_V3.3_*-*_A0A072U2K9_MEDTR)
Solyc06g05166 0.1.1	2,658160718	Early_flowering_4_(AHRD_V3.3_***_A0A059V8X7_9ROSI)
Solyc12g04953 0.2.1	2,681190349	Phosphoinositide_phosphatase_family_protein_(AHRD_V3.3_*-*_B9GXR7_POPTR)
Solyc10g05405 0.2.1	6,946719724	Kinase_putative_(AHRD_V3.3_***_B9RH93_RICCO)
Solyc10g01833 0.2.1	8,099909553	Retrovirus-related_Pol_polyprotein_from_transposon_TNT_1-94_(AHRD_V3.3_*- *_A0A151TG49_CAJCA)

Solyc02g07239 3.1.1	6,96556089	Receptor-kinase_putative_(AHRD_V3.3_***_B9RVA8_RICCO)
Solyc03g09338 0.2.1	2,20445921	protein_kinase_family_protein_(AHRD_V3.3_***_AT1G51870.2)
Solyc01g00921 5.1.1	2,857293604	Heat_shock_family_protein_(AHRD_V3.3_***_B9HQH9_POPTR)
Solyc09g09002 0.3.1	2,205759494	Germin-like_protein_1_(AHRD_V3.3_***_A8QK89_VITVI)
Solyc02g07041 0.2.1	2,405329371	Disease_resistance_protein_putative_(AHRD_V3.3_***_Q6L432_SOLDE)
Solyc04g07200 0.3.1	2,182336073	Chitinase_(AHRD_V3.3_***_A0A1B1HY32_9APIA)
Solyc06g05432 0.1.1	2,690758759	Dirigent_protein_(AHRD_V3.3_***_K4C6B8_SOLLC)
Solyc07g00776 0.3.1	2,154841917	defensin-like_protein
Solyc10g05519 0.1.1	5,216357243	Dirigent_protein_(AHRD_V3.3_***_K4D1B2_SOLLC)
Solyc10g05520 0.1.1	2,06850204	Dirigent_protein_(AHRD_V3.3_***_K4D1B3_SOLLC)
Solyc10g08610 0.2.1	2,406338004	Proteinase_inhibitor_(AHRD_V3.3_***_A0A0R4WSF0_TOBAC)
Solyc04g01027 0.1.1	2,350516375	Dirigent_protein_(AHRD_V3.3_***_K4BPI1_SOLLC)
Solyc05g05478 0.2.1	2,061752013	Dirigent_protein_(AHRD_V3.3_***_M1C5D5_SOLTU)
Solyc08g01397 0.2.1	4,300639718	Disease_resistance_protein_(AHRD_V3.3_***_W9RZV6_9ROSA)
Solyc09g09121 0.3.1	2,186005893	Dirigent_protein_(AHRD_V3.3_***_K4CWE7_SOLLC)
Solyc02g06775 0.3.1	6,915338794	Carbonic_anhydrase_(AHRD_V3.3_***_B1VK36_SOLLC)
Solyc09g00983 0.3.1	2,012604497	Carbonic_anhydrase_family_protein_(AHRD_V3.3_***_D7LUA2_ARALL)
Solyc03g09575 0.2.1	8,473303395	Chlorophyllide_A_oxygenase_(AHRD_V3.3_***_G7I8G8_MEDTR)
Solyc09g08260 0.2.1	7,534363796	Chlorophyllase_(AHRD_V3.3_***_F1BPW6_SOLPN)
Solyc01g10543 5.1.1	2,773992398	ABC_transporter_G_family_member_(AHRD_V3.3_***_A0A0K9NWI5_ZOSMR)
Solyc04g01020 0.1.1	4,543196954	ABC_transporter_G_family_member_(AHRD_V3.3_***_A0A0K9NPK2_ZOSMR)
Solyc07g06412 0.2.1	7,634398087	ABC_transporter_B_family_protein_(AHRD_V3.3_***_G7JF16_MEDTR)
Solyc00g00713 0.3.1	3,654367702	Amino_acid_transporter_putative_(AHRD_V3.3_***_B9S6V9_RICCO)
Solyc03g01344 0.3.1	2,381868974	Amino_acid_transporter_putative_(AHRD_V3.3_***_B9T0U1_RICCO)
Solyc08g07782 0.3.1	3,271010027	Cationic_amino_acid_transporter_putative_(AHRD_V3.3_***_A0A061G4V0_THECC)
Solyc03g00598 0.3.1	2,189022209	NOD26-like_intrinsic_protein_1.1
Solyc02g08890 0.3.1	2,149465776	Nucleotide-sugar_transporter_family_protein_(AHRD_V3.3_***_AT3G17430.2)
Solyc05g04739 0.1.1	2,142589642	Mitochondrial_import_receptor_subunit_TOM7-1_(AHRD_V3.3_***_TOM7A_SOLTU)

Solyc11g01853 5.1.1	6,81019775	Metal_transporter_putative_(AHRD_V3.3_**_B9S2W4_RICCO)
Solyc03g02519 0.4.1	16,80814437	anthocyanin_permease
Solyc08g00743 0.2.1	2,31645093	NIT2
Solyc08g06699 0.3.1	3,421204244	Potassium_channel_(AHRD_V3.3_***_O24382_SOLTU)

Downregulated DEGs		
Gene ID	Log ₂ FC	Gene description
Solyc02g09430 0.3.1	-2,609639	ACT_domain-containing_protein_(AHRD_V3.3_**_A0A103Y4L4_CYNCS)
Solyc05g00961 0.1.1	-2,1242823	Alpha/beta-Hydrolases_superfamily_protein_(AHRD_V3.3_***_A0A061EB00_THECC)
Solyc08g07503 4.1.1	-6,6720112	Pectinacetylesterase_family_protein_(AHRD_V3.3_**_AT5G45280.2)
Solyc02g06730 0.2.1	-2,8544233	Structural_maintenance_of_chromosomes_protein_(AHRD_V3.3_**_A0A0K9Q8I9_SPIOL)
Solyc10g08646 0.2.1	-3,6609937	Actin_(AHRD_V3.3_***_ACT1_TOBAC)
Solyc01g10478 0.3.1	-2,7548188	Vacuolar_iron_transporter_family_protein_(AHRD_V3.3_***_A0A061DXK2_THECC)
Solyc03g09683 0.3.1	-2,3208247	Pentatricopeptide_repeat-containing_protein_putative_(AHRD_V3.3_***_B9S1X8_RICCO)
Solyc08g06526 5.1.1	-6,317298	BED_zinc_finger_hAT_family_dimerization_domain_putative_(AHRD_V3.3_***_A0A061FD65_THECC)
Solyc01g08046 0.3.1	-2,0955519	pyruvate_orthophosphate_dikinase_(AHRD_V3.3_***_AT4G15530.6)
Solyc04g00990 0.3.1	-2,019337	phosphoenolpyruvate_carboxylase_kinase
Solyc08g07833 0.3.1	-4,5759073	2-oxoglutarate_(2OG)_and_Fe(II)-dependent_oxygenase_superfamily_protein_(AHRD_V3.3_***_AT4G16765.6)
Solyc02g02289 0.2.1	-3,7512686	Fatty_acid_2-hydroxylase_(AHRD_V3.3_***_A0A1D1ZHT2_9ARAE)
Solyc01g10261 0.3.1	-3,6079164	Ferric_reduction_oxidase_7_(AHRD_V3.3_***_W9RRH7_9ROSA)
Solyc08g00708 0.3.1	-2,0407175	Type_I_inositol-1,4,5-trisphosphate_5-phosphatase_(AHRD_V3.3_***_G7JZV2_MEDTR)
Solyc02g06522 0.3.1	-2,1410822	Cytochrome_P450_(AHRD_V3.3_***_A0A103Y530_CYNCS)
Solyc09g06615 0.1.1	-4,5745465	Cytochrome_P450_putative_(AHRD_V3.3_***_B9S4U5_RICCO)
Solyc12g09662 0.1.1	-2,382962	GDSL_esterase
Solyc12g01095 0.2.1	-7,8920961	NADP-dependent_alkenal_double_bond_reductase_(AHRD_V3.3_***_A0A072TLD5_MEDTR)
Solyc07g06302 0.2.1	-2,8339049	Early_nodulin-like_protein_(AHRD_V3.3_**_A0A072VCH6_MEDTR)
Solyc03g09744 0.3.1	-3,6912947	Dehydrogenase/reductase_SDR_family_protein_7-like_protein_(AHRD_V3.3_***_A0A0B0P5K8_GOSAR)
Solyc04g01620 0.1.1	-2,9616849	Glycosyltransferase_(AHRD_V3.3_***_Q8RXA4_SOLLC)

Solyc04g01621 0.3.1	-2,1701291	Glycosyltransferase_(AHRD_V3.3_***_K4BQC1_SOLLC)
Solyc05g05389 0.2.1	-2,0325692	Glycosyltransferase_(AHRD_V3.3_***_K4C268_SOLLC)
Solyc12g00643 0.2.1	-3,8352398	Glycosyltransferase_(AHRD_V3.3_***_K4DBI0_SOLLC)
Solyc12g01385 0.2.1	-2,0368037	Core-2/I-branching_beta-1,6-N-acetylglucosaminyltransferase_family_protein_(AHRD_V3.3_***_F4JIW2_ARATH)
Solyc00g01974 0.1.1	-2,0963003	ATPase_subunit_8_(AHRD_V3.3_***_A0A0K1ZF72_SOLCA)
Solyc00g01313 7.1.1	-2,246679	NADH_dehydrogenase_subunit_2_(AHRD_V3.3_**_D5I397_CITLA)
Solyc11g03057 0.1.1	-2,7253851	LOW_QUALITY:NADH-ubiquinone_oxidoreductase_chain_4_(AHRD_V3.3_**_NU4M_WHEAT)
Solyc05g00528 0.3.1	-2,0891929	Poly_[ADP-ribose]_polymerase_(AHRD_V3.3_***_K4BW59_SOLLC)
Solyc09g00719 0.3.1	-2,2183152	Thioredoxin_superfamily_protein_(AHRD_V3.3_***_AT2G37240.1)
Solyc07g05270 7.1.1	-2,3279228	Formin-like_protein_(AHRD_V3.3_**_G8IRK0_SOLLC)
Solyc00g01318 1.1.1	-2,3253355	S-adenosyl-L-methionine-dependent_methyltransferases_superfamily_protein_(AHRD_V3.3_**_AT1G08125.3)
Solyc00g01486 0.1.1	-3,7941237	LOW_QUALITY:Pectin_lyase-like_superfamily_protein_(AHRD_V3.3_**_AT3G61490.5)
Solyc00g01999 0.1.1	-2,328655	LOW_QUALITY:Leucine-rich_repeat_receptor-like_protein_kinase_family_protein_(AHRD_V3.3_**_AT2G33170.2)
Solyc00g02000 0.1.1	-2,1317005	LOW_QUALITY:Mitogen-activated_protein_kinase_(AHRD_V3.3_**_A0A0E0HQW2_ORYNI)
Solyc00g02209 0.2.1	-2,4302695	Formyltetrahydrofolate_deformylase_1_mitochondrial_(AHRD_V3.3_**_PURU1_ARATH)
Solyc00g02358 0.1.1	-3,0079174	Thioredoxin_superfamily_protein_(AHRD_V3.3_**_AT1G07700.4)
Solyc00g05254 0.1.1	-4,002873	LOW_QUALITY:RING/FYVE/PHD_zinc_finger_superfamily_protein_(AHRD_V3.3_**_AT3G02890.4)
Solyc00g09455 0.1.1	-2,822964	LOW_QUALITY:U-box_domain-containing_protein_14_(AHRD_V3.3_**_PUB14_ARATH)
Solyc00g10855 0.1.1	-2,1219066	LOW_QUALITY:Homeodomain-like_superfamily_protein_putative_isoform_1_(AHRD_V3.3_**_A0A061G387_THECC)
Solyc00g12600 0.1.1	-2,1426177	NADH-ubiquinone_oxidoreductase_chain_2_(AHRD_V3.3_**_NU2M_BETVU)
Solyc00g12601 0.1.1	-2,1281241	LOW_protein:NADH-ubiquinone_oxidoreductase_chain-like_protein_(AHRD_V3.3_**_ATMG01330.1)
Solyc00g32043 0.1.1	-2,033171	LOW_QUALITY:Vascular_endothelial_growth_factor_receptor_2_(AHRD_V3.3_**_A0A1D1YH50_9ARAE)
Solyc01g10442 0.1.1	-4,4611938	LOW_QUALITY:guanosine_nucleotide_diphosphate_dissociation_inhibitor_1_(AHRD_V3.3_**_AT2G44100.1)
Solyc01g10746 0.2.1	-3,4280364	LOW_QUALITY:guanosine_nucleotide_diphosphate_dissociation_inhibitor_1_(AHRD_V3.3_**_AT2G44100.1)
Solyc01g10992 0.2.1	-2,4106137	LOW_QUALITY:Dehydrin_protein_(AHRD_V3.3_***_V9M5C3_MANES)
Solyc01g11068 2.1.1	-2,142939	DNA_mismatch_repair_protein_MLH3-like_protein_(AHRD_V3.3_**_A0A0X7YCF6_SOLTU)
Solyc01g11345 0.2.1	-8,3519846	LOW_QUALITY:DEAD-box_ATP-dependent_RNA_helicase_29_(AHRD_V3.3_**_RH29_ORYSI)

Solyc02g02290 0.3.1	-4,9897843	fatty_acid_hydroxylase_2_(AHRD_V3.3_--*_AT4G20870.2)
Solyc02g08428 5.1.1	-2,0542217	Nuclear_pore_complex_protein_(AHRD_V3.3_--*_W9SV07_9ROSA)
Solyc02g08431 0.2.1	-5,6600325	CTP_synthase_(AHRD_V3.3_--*_M1BXT3_SOLTU)
Solyc02g08431 5.1.1	-3,8260203	embryo_defective_2410_(AHRD_V3.3_--*_AT2G25660.4)
Solyc02g08594 0.3.1	-3,8611741	Calcium-dependent_phosphotriesterase_superfamily_protein_(AHRD_V3.3_--*_AT3G59530.3)
Solyc02g09139 0.3.1	-2,2022791	cold-regulated_protein_(AHRD_V3.3_***_AT3G03341.1)
Solyc03g09876 0.2.1	-5,1094695	Kunitz-type_protease_inhibitor-like_protein_(AHRD_V3.3_***_Q2XPY0_SOLTU)
Solyc04g01773 0.1.1	-2,2388633	LOW_QUALITY:30S_ribosomal_protein_S2_chloroplastic_(AHRD_V3.3_--*_RR2_LEMMI)
Solyc04g04922 6.1.1	-4,1808087	Pentatricopeptide_repeat-containing_protein_mitochondrial_(AHRD_V3.3_--*_A0A0B2Q4X7_GLYSO)
Solyc04g05149 0.3.1	-2,1781499	essential_meiotic_endonuclease_1B_(AHRD_V3.3_--*_AT2G22140.1)
Solyc04g07735 7.1.1	-2,4188654	HAUS_augmin-like_complex_subunit_(AHRD_V3.3_--*_AT5G38880.1)
Solyc05g00949 0.2.1	-2,0951773	DNA_polymerase_epsilon_catalytic_subunit_(AHRD_V3.3_--*_AT2G27120.3)
Solyc05g01280 5.1.1	-2,227419	transmembrane_protein_(AHRD_V3.3_***_AT3G11810.1)
Solyc05g02366 0.2.1	-6,3207835	LOW_QUALITY:Transposase_(AHRD_V3.3_--*_F8UTT6_SOLDE)
Solyc05g02377 5.1.1	-4,3489232	NADH-quinone_oxidoreductase_protein_(AHRD_V3.3_--*_A0A072V639_MEDTR)
Solyc05g02399 0.2.1	-6,4691926	LOW_QUALITY:Leucine-rich_receptor-like_protein_kinase_family_protein_(AHRD_V3.3_--*_AT1G09970.2)
Solyc05g02419 0.3.1	-7,4968297	Chlorophyll_synthase_(AHRD_V3.3_--*_C3W4Q2_TOBAC)
Solyc06g01800 4.1.1	-3,5201374	tetraspanin10_(AHRD_V3.3_--*_AT1G63260.6)
Solyc06g06885 0.3.1	-2,4379627	DCD_(Development_and_Cell_Death)_domain_protein_(AHRD_V3.3_--*_AT5G61910.5)
Solyc06g07171 0.1.1	-6,4459109	LOW_QUALITY:ARM_repeat_superfamily_protein_(AHRD_V3.3_--*_AT1G71020.1)
Solyc07g01839 0.2.1	-2,1243289	RING-H2_finger_protein_2B_(AHRD_V3.3_--*_AT2G01150.2)
Solyc07g05270 3.1.1	-6,5463871	F-box_family_protein_(AHRD_V3.3_--*_AT1G25211.1)
Solyc07g05560 0.1.1	-4,0489273	LOW_QUALITY:Phytochrome_a_(AHRD_V3.3_--*_PHYA_SORBI)
Solyc08g00863 0.3.1	-3,3407965	beta-carotene_isomerase_D27_(AHRD_V3.3_***_AT1G64680.2)
Solyc08g06663 0.2.1	-2,1453095	LOW_QUALITY:F-box/LRR-repeat_protein_4_(AHRD_V3.3_--*_A0A1D1XF78_9ARAE)
Solyc08g07503 2.1.1	-7,3193778	Leaf_senescence_protein-like_(AHRD_V3.3_--*_Q6YWS1_ORYSJ)
Solyc09g03703 0.1.1	-2,0157229	LOW_QUALITY:Sister-chromatide_cohesion_protein_3_(AHRD_V3.3_--*_D7LHE4_ARALL)
Solyc09g05631 0.1.1	-4,9862972	LOW_QUALITY:Maturase_(AHRD_V3.3_--*_O79414_SOLTU)

Solyc09g05939 0.2.1	-2,2540039	Transducin_family_protein_/WD-40_repeat_family_protein_(AHRD_V3.3_--*_AT5G43930.3)
Solyc09g08949 0.3.1	-5,2113782	Proteinase_inhibitor_I_(AHRD_V3.3_***_Q3S492_SOLTU)
Solyc11g03937 0.2.1	-2,3730809	LOW_QUALITY:Glycosyl_hydrolase_superfamily_protein_(AHRD_V3.3_--*_AT3G15800.1)
Solyc11g05644 0.1.1	-4,6164033	LOW_QUALITY:ureide_permease_2_(AHRD_V3.3_--*_AT2G03530.4)
Solyc12g00913 0.2.1	-4,2498225	LURP-one-like_protein_(AHRD_V3.3_***_G7IGE6_MEDTR)
Solyc12g04961 3.1.1	-3,692418	Glycosyltransferase_(AHRD_V3.3_--*_M1CLQ4_SOLTU)
Solyc02g08256 0.1.1	-2,3741905	CTP_synthase_family_protein_(AHRD_V3.3_***_AT4G02120.5)
Solyc02g08430 0.1.1	-2,9655268	CTP_synthase_(AHRD_V3.3_***_M1B2B1_SOLTU)
Solyc06g05137 0.1.1	-2,933645	LOW_QUALITY:Protein_Ycf2_(AHRD_V3.3_***_YCF2_SOLLC)
Solyc02g02122 0.1.1	-2,1335082	Subtilisin-like_protease_(AHRD_V3.3_***_W9R4Z9_9ROSA)
Solyc12g09689 0.1.1	-2,3655241	F-box_protein_PP2_(AHRD_V3.3_***_A0A059PC27_CICAR)
Solyc03g00696 0.3.1	-2,6988129	Protein_phosphatase_2C_family_protein_(AHRD_V3.3_***_AT5G51760.1)
Solyc04g07710 0.3.1	-2,0287923	Poly_[ADP-ribose]_polymerase_(AHRD_V3.3_***_K4BUE7_SOLLC)
Solyc00g18044 0.2.1	-2,7828429	50S_ribosomal_protein_L20_chloroplastic_(AHRD_V3.3_***_A0A0A1IRS6_9LAMI)
Solyc02g08595 0.3.1	-3,2672291	cell_wall_protein_X77373
Solyc11g02111 0.2.1	-2,7087162	NAD(P)H-quinone_oxidoreductase_subunit_2_chloroplastic_(AHRD_V3.3_***_NU2C_ANACO)
Solyc06g00876 0.1.1	-3,0561159	Glutaredoxin_(AHRD_V3.3_***_B3F8F2_SOLTU)
Solyc03g09363 0.2.1	-2,0349852	Pentatricopeptide_repeat-containing_protein_(AHRD_V3.3_***_A0A0K9PNP9_ZOSMR)
Solyc09g01007 0.1.1	-2,0108919	Pentatricopeptide_repeat_superfamily_protein_(AHRD_V3.3_***_A0A061F1A8_TH_ECC)
Solyc03g11958 0.1.1	-2,6606418	Solanum_lycopersicum_Cytokinin_Response_Factor_9
Solyc08g06113 0.3.1	-2,068812	BZIP_transcription_factor_(AHRD_V3.3_***_L0MZN5_MALDO)
Solyc00g01480 0.1.1	-2,1798919	Zinc_finger_family_protein_(AHRD_V3.3_***_B9GFF8_POPTR)
Solyc02g06296 0.3.1	-3,5392765	Homeobox-leucine_zipper_family_protein_(AHRD_V3.3_***_B9HET5_POPTR)
Solyc07g05272 0.3.1	-3,6103571	MADS-box_transcription_factor_(AHRD_V3.3_***_A0A072UYG7_MEDTR)
Solyc01g09576 0.3.1	-6,3166078	Glycosyltransferase_(AHRD_V3.3_***_K4AZW5_SOLLC)
Solyc04g05093 0.3.1	-2,0729539	violaxanthin_de-epoxidase
Solyc05g01018 0.3.1	-2,221216	Carotenoid_isomerase_(AHRD_V3.3_***_A0A077EPD5_TOBAC)
Solyc02g03050 5.1.1	-3,6010218	Calcium-transporting_ATPase_(AHRD_V3.3_***_K4D2H1_SOLLC)

Solyc08g06293 0.2.1	-6,6778796	Calmodulin_binding_protein,_putative_(AHRD_V3.3_***_B9RQR7_RICCO)
Solyc01g01048 0.3.1	-2,1235749	LOV/LOV_protein_(AHRD_V3.3_***_E3WH16_SOLLC)
Solyc11g06996 0.2.1	-2,5678708	RLK-1
Solyc07g05561 0.2.1	-2,055605	Disease_resistance_protein_(TIR-NBS-LRR_class),_putative_(AHRD_V3.3_***_G7IQ97_MEDTR)
Solyc02g08682 0.3.1	-3,6678967	chloroplast_carbonic_anhydrase
Solyc01g07989 0.3.1	-3,6371142	NOD26-like_intrinsic_protein_7.1
Solyc07g01468 0.3.1	-2,8823282	sodium_transporter_HKT1,2

Appendix II. Gene Ontology Enrichment (GOE) results obtained using gProfiler. GO categories resulted: Molecular Function (MF), Biological Process (BP) and Cellular Component (CC).

Upregulated DEGs									
Source	term_name	term id	adjusted p_value	negative log10 of adjusted p-value	term_size	query size	intersection size	effective domain size	intersections
GO:MF	serine-type endopeptidase inhibitor activity	GO:0004867	5,92683E-06	5,227177212	33	165	7	17440	SOLYC03G020080.3.1, SOLYC03G020030.3.1, SOLYC07G054720.2.1, SOLYC11G020960.2.1, SOLYC08G080630.3.1, SOLYC10G086090.2.1, SOLYC10G086100.2.1
GO:MF	endopeptidase inhibitor activity	GO:0004866	0,000604961	3,218272396	63	165	7	17440	SOLYC03G020080.3.1, SOLYC03G020030.3.1, SOLYC07G054720.2.1, SOLYC11G020960.2.1, SOLYC08G080630.3.1, SOLYC10G086090.2.1, SOLYC10G086100.2.1
GO:MF	peptidase inhibitor activity	GO:0030414	0,000604961	3,218272396	63	165	7	17440	SOLYC03G020080.3.1, SOLYC03G020030.3.1, SOLYC07G054720.2.1, SOLYC11G020960.2.1, SOLYC08G080630.3.1, SOLYC10G086090.2.1, SOLYC10G086100.2.1
GO:MF	endopeptidase regulator activity	GO:0061135	0,000673893	3,171409232	64	165	7	17440	SOLYC03G020080.3.1, SOLYC03G020030.3.1, SOLYC07G054720.2.1, SOLYC11G020960.2.1, SOLYC08G080630.3.1, SOLYC10G086090.2.1, SOLYC10G086100.2.1
GO:MF	peptidase regulator activity	GO:0061134	0,000749263	3,125365518	65	165	7	17440	SOLYC03G020080.3.1, SOLYC03G020030.3.1, SOLYC07G054720.2.1, SOLYC11G020960.2.1, SOLYC08G080630.3.1, SOLYC10G086090.2.1, SOLYC10G086100.2.1

GO:MF	beta-glucosidase activity	GO:0008422	0,007657708	2,115901211	19	165	4	17440	SOLYC06G073760.3.1, SOLYC01G059965.1.1, SOLYC10G045240.2.1, SOLYC12G040640.2.1
GO:MF	naringenin-chalcone synthase activity	GO:0016210	0,025420945	1,59480831	2	165	2	17440	SOLYC05G053550.3.1, SOLYC09G091510.3.1
GO:MF	glucosidase activity	GO:0015926	0,028049474	1,552075282	26	165	4	17440	SOLYC06G073760.3.1, SOLYC01G059965.1.1, SOLYC10G045240.2.1, SOLYC12G040640.2.1
GO:MF	enzyme inhibitor activity	GO:0004857	0,030127162	1,521041774	196	165	9	17440	SOLYC05G054360.3.1, SOLYC03G020080.3.1, SOLYC03G020030.3.1, SOLYC07G054720.2.1, SOLYC11G020960.2.1, SOLYC08G080630.3.1, SOLYC10G0806090.2.1, SOLYC10G018330.2.1, SOLYC10G086100.2.1
GO:BP	flavonoid biosynthetic process	GO:0009813	0,000486516	3,3129029	9	123	4	13460	SOLYC05G053550.3.1, SOLYC09G091510.3.1, SOLYC02G083860.3.1, SOLYC02G085020.3.1
GO:BP	flavonoid metabolic process	GO:0009812	0,00080513	3,094134012	10	123	4	13460	SOLYC05G053550.3.1, SOLYC09G091510.3.1, SOLYC02G083860.3.1, SOLYC02G085020.3.1
GO:CC	extracellular region	GO:0005576	1,85841E-05	4,730858973	352	115	17	11083	SOLYC01G106650.3.1, SOLYC03G093390.3.1, SOLYC05G052245.1.1, SOLYC08G077910.3.1, SOLYC09G010860.3.1, SOLYC11G011210.2.1, SOLYC02G062510.3.1, SOLYC02G077300.2.1, SOLYC04G080760.3.1, SOLYC01G110110.3.1, SOLYC02G076710.3.1, SOLYC08G080630.3.1, SOLYC07G006570.3.1,

									SOLYC06G054320.1.1, SOLYC10G055190.1.1, SOLYC10G055200.1.1, SOLYC04G010270.1.1
GO:CC	Casparian strip	GO:0048226	0,049850003	1,302334811	3	115	2	11083	SOLYC09G010200.3.1, SOLYC10G083250.2.1
KEGG	Flavonoid biosynthesis	KEGG:00941	0,001610898	2,792932077	36	31	5	2506	SOLYC12G088170.2.1, SOLYC05G053550.3.1, SOLYC09G091510.3.1, SOLYC02G083860.3.1, SOLYC09G082660.3.1
KEGG	Phenylpropanoid biosynthesis	KEGG:00940	0,035876145	1,445194224	103	31	6	2506	SOLYC06G073760.3.1, SOLYC02G062510.3.1, SOLYC02G077300.2.1, SOLYC04G080760.3.1, SOLYC12G088170.2.1, SOLYC09G082660.3.1

Downregulated DEGs									
Source	term_name	term id	adjusted p_value	negative log10 of adjusted p-value	term_size	query size	intersection size	effective domain size	intersections
GO:MF	transferase activity, transferring glycosyl groups	GO:0016757	0,029442428	1,531026376	529	51	8	17440	SOLYC04G016200.1.1, SOLYC04G016210.3.1, SOLYC05G053890.2.1, SOLYC12G006430.2.1, SOLYC12G013850.2.1, SOLYC05G005280.3.1, SOLYC04G077100.3.1, SOLYC01G095760.3.1

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