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Maastricht University

Faculty of Medicine and Life Sciences
School for Life Sciences
Master of Biomedical Sciences

Master's thesis

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Esteban Suls

Thesis presented in fulfillment of the requirements for the degree of Master of Biomedical Sciences, specialization Environmental Health Sciences

SUPERVISOR :
Prof. dr. Nele HOREMANS
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Ionising radiation exposure: how it affects gene expression and pine tree morphology*

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*Running title: *Methylation, chromatin and radiation in pines*

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ABSTRACT

Ionising radiation continues to pose an environmental threat to human and non-human biota, for example at sites of nuclear disasters like the Chornobyl and Fukushima exclusion zones. Pine trees are especially sensitive to radiation, and therefore of high interest for research on radioactive contamination of the environment. To better understand the radiation response of the highly sensitive pine tree – commonly found at the Chornobyl and Fukushima exclusion zones, we investigated whether gene expression is altered by radiation (dose rate: 682 µGy/h) in a 10-week laboratory exposure experiment of young *Pinus sylvestris* seedlings.

RT-qPCR revealed a lower expression of METHYLTRANSFERASE 1 (MET1) in roots at week 10 of the cultivation. Phenotypically, the roots of irradiated seedlings weighed less at week 10 compared to control roots. Moreover, at weeks 4 and 6, the total length of plants was significantly lower for the irradiated group. Furthermore, exposure to ionising radiation seemed to alter the morphology of the shoot apex, although no changes in gene expression were observed in the shoot tissue.

Aside from the molecular and phenotypical analyses of irradiated samples, attempts were made to develop a protocol for flow cytometry imaging of stained pine nuclei to investigate differences in chromatin condensation in irradiated samples. Using fluorescence microscopy we confirmed nuclei were successfully extracted and stained. However, we were not able to visualise the nuclei using a flow cytometric approach.

In conclusion, results here may indicate changes in methylation in irradiated seedlings, and its potential role in morphological changes will be discussed here.

Keywords: *methylation enzymes, pine trees, gamma radiation, flow cytometry, chromatin condensation*

INTRODUCTION

Pines in radioactive areas

Ever since the nuclear accidents at the Fukushima Dai-Ichi power plant in 2011 and the Chornobyl disaster of 1986, many efforts have been made to establish the scale of radionuclide contamination in the affected areas (1, 2, 3). Despite these efforts made in the Fukushima exclusion zone (FEZ) and Chornobyl exclusion zone (CEZ), many effects of long-term radionuclide exposure remain unknown, especially regarding non-human biota in the contaminated areas. It is well-known how the acute effects of radionuclide exposure impact the health of plants, yet the effects of long-term chronic exposure remain poorly understood (4). The fact that these two locations are unique opportunities to compare the effects of radionuclide exposure in the field with laboratory exposure experiments also contributes to the gap in knowledge regarding the effects of long-term radionuclide exposure.

In the past, common non-model organisms in the FEZ and CEZ have been omitted as the first choice for gene expression analyses and other studies on the responses to radionuclide exposure, because they are naturally more difficult to work with. Commonly found organisms in these areas that react intensely to radiation might, however, serve as a bioindicator for radiation damage or radionuclide contamination of the environment (5). One such non-model organism is the pine tree, which is of high interest in radionuclide research due to its relatively high sensitivity to ionising radiation in contrast to other plants such as deciduous trees and grasses (5, 6, 7). It has been reported that Scots pine (*Pinus sylvestris*) has a

median lethal dose (LD_{50}) of 5 – 20 Gy, while for many angiosperms this value reaches in the hundreds of Gy (8). Shortly after the Chornobyl accident, massive death of pine trees was reported in what is now known as the Red Forest, while deciduous species in the same area survived despite an early loss of leaves and some damage to woody tissue (9, 10). Additionally, pine trees have a large genome compared to deciduous trees, which makes them especially vulnerable to radiation-induced DNA damage. Pine trees were also included in the list of reference animals and plants used to set goals for radiation protection of non-human biota by the International Commission on Radiation Protection (11). They are native to both the FEZ and the CEZ, harbouring important ecological roles such as sequestering carbon (as carbon reservoir), and interactions with soil microbes and fungi (mycorrhizae) (12, 13, 14). After long-term exposure to ionising radiation following the Fukushima nuclear accident, local *Pinus densiflora* (Japanese red pine) trees showed morphological abnormalities such as the loss of apical dominance; similarly, *Pinus sylvestris* native to zones outside of the Red Forest in the CEZ showed morphological abnormalities in the form of cancellation of apical dominance (15, 16, 17). Yet, after 30 years of observations at the CEZ site, and over 10 years of observations at the FEZ site, still no mechanistic explanation was found for the display of these morphological abnormalities in pine trees (16). Instead, in recent years, additional studies have emerged on other organisms such as bumblebees, herbaceous plants, domestic dogs, *Linum usitatissimum* (flax), *Drosophila melanogaster* (fruit fly), and many other organisms (5, 6, 18, 19, 20, 21).

Epigenetic regulation

Since ionising radiation has been described to disturb gene expression by inducing DNA damage, gene expression may be altered in morphologically abnormal trees (22). Specifically, the expression of genes encoding enzymes related to epigenetic regulation is of particular interest as certain genes have been shown to be involved in defensive responses to stress (23), but they are also important for the growth and development of plants such as phytohormone pathways. For example, it has been reported that epigenetic regulatory enzymes are an important regulatory system of the auxin pathway (24, 25, 26). Because of this direct link between gene expression and morphological abnormalities – such as the loss of apical dominance possibly caused by a disturbance of the auxin pathway, the epigenetic aspect of this link is of high interest to unravel the exact effects of ionising radiation on pine trees. Additionally, plant responses to stress seem to be regulated at the molecular level by changes in gene expression too (4, 27). DNA methylation of certain promoter regions may cause directed silencing or activation of gene expression and hence DNA methylation might play a regulatory role in the observed changes in gene expression (28). Furthermore, a recent review has suggested that DNA methylation is closely involved in transferring the response to ionising radiation from one generation to the next, but also that epigenetic changes occurred following exposure to ionising radiation (9). Although much is still to be learnt about how organisms react to and store information concerning previous exposures, it is likely that epigenetic mechanisms are major contributors to this process (4).

Another epigenetic regulatory process that alters the 3D structure of DNA called chromatin condensation is also crucial in the protection of the genome from radiation-induced damage by compacting the DNA closely together around histones as heterochromatin (29). In contrast, euchromatin is loosely wrapped around histones and more available for gene expression and epigenetic regulators like methyltransferases, but also more prone to double-strand breaks (DSBs) and other forms of radiation-induced DNA damage (29, 30, 31). For example, Takata et al. found a 16-fold greater damage suppression in condensed chromatin compared to decondensed chromatin. Even at the relatively high level of ionising radiation of 5 Gy, the same damage suppression effect of condensed chromatin was found at a 5-fold rate compared to decondensed chromatin (29). It is suspected that heterochromatin has fewer water molecules associated per chromatin, which allows less generation of reactive radicals such as superoxide ($^{\bullet}O_2^-$) and hydroxyl radicals ($^{\bullet}OH$).

The role of DNA methylation enzymes

DNA methylation at the 5' position of cytosine contributes to the epigenetic regulation of gene expression by directly repressing transcription

through methylation of promotor regions, but also by altering the chromatin structure to change the accessibility of genetic information (23). For example, hypermethylation of CG-rich promoters can be associated with repression of gene expression. This DNA methylation can be carried out *de novo* or via maintenance methyltransferase enzymes (9, 32). The methylation enzymes involved in this process are more diversified in plants than in animals, with more extensive effects on a wider sequence diversity. This multitude of different methylation enzymes in plants means some have no analogues in animals, further substantiating the diversification of methylation enzymes in plants (33, 34).

Contrary to DNA methylation in animals, which is dominated by methylation at CG sites, methylation in plants can also occur at non-CG sites like CHG and CHH sites (H = A, T, or C) (9, 35, 36). In animals, *de novo* methylation and maintenance are carried out by DNA METHYLTRANSFERASE 3 (DNMT3) protein family members and DNMT1 respectively. In plants, homologues of DNMT3, DOMAINS REARRANGED METHYLTRANSFERASE 1/2 (DRM1/2) are responsible for *de novo* methylation while maintenance of methylation at CG sites is managed by DNA METHYLTRANSFERASE 1 (MET1) which is a homologue of DNMT1 in animals. Another important enzyme suspected to be involved in the maintenance of methylation is REPRESSOR OF SILENCING 1 (ROS1), although it is more commonly known to demethylate promoter regions and remove methyl groups from improperly-methylated cytosines (37). In addition, CHROMO-METHYLASE 3 (CMT3) is responsible for the maintenance of methylation at plant-specific CHG sites, along with DRM1 and DRM2 for CHH sites (9, 38). Notably, CHA sites have a threefold higher methylation frequency compared to CHC and CHT sites, while CHGs in the context of CTGG sites are methylated 6.5 times more frequently than CCGC sites, CGs in the context of a GCGG site are methylated twice as frequently as ACGT sites. This illustration of the methylation frequency diversity between the different contexts of DNA methylation further drives home the complexity of DNA methylation and DNA methyltransferase preferences beyond the CG, CHG, and CHH contexts in plants (34). Given the complexity of this topic and the non-model nature of pine trees, there are still many mysteries surrounding DNA methylation that elude us, especially in the context of radionuclide exposure.

DNA methylation and chromatin condensation
DNA methylation has been described to have a direct influence on the hormone metabolism in *Arabidopsis thaliana* (9, 25). A recent study on *A. thaliana* found that a triple mutant of the genes *drm1*, *drm2*, and *cmt3* transcribing for methylation enzymes causes several developmental abnormalities from the embryo to the adult plant stage (25). It was found that not only auxin accumulation and distribution were affected in the mutant compared to the wild type, but also the expression of genes that regulate the auxin hormone pathways was affected (25). Furthermore, ionising radiation has been described to disturb the methylation process (9, 39). Therefore, we hypothesise DNA methylation plays an important role as a linking mechanism between radionuclide exposure and potential hormonal changes that could cause the morphological abnormalities in irradiated pine trees as observed in the field.

In addition to this, the organisation of chromatin plays an important role in protection against radiation-induced damage (29). Therefore, we wanted to examine if chromatin condensation is altered following exposure to ionising radiation. It is also possible the expression of SIRTUIN 1 and 2 (SRT1/2) may be altered. These enzymes are involved in the regulation of many aspects of chromatin biology such as transcription and recombination, but most notably genome stability and chromatin condensation (40). Primarily by modifying histones through deacylation, they modulate the direct accessibility of the DNA for transcription (41). We believe their activity may be altered due to stressors like radionuclide exposure. Furthermore, changes in their gene expression may be directly linked to chromatin condensation. A higher expression of deacylating sirtuins results in fewer acyl groups bound to histone octamers, allowing the DNA around it to bind more closely, thus packing the chromatin densely in a heterochromatin structure (41).

This research aims to explore the consequences of long-term radionuclide exposure on the expression of genes encoding DNA methylation enzymes and

sirtuins in pine trees. We will also closely examine differences in phenotype between exposed and unexposed plants cultivated in a controlled setting. We expect to observe changes in the phenotype of irradiated pine trees. The expression of genes encoding epigenetically relevant enzymes will be analysed to determine whether these changes in expression could contribute to potential morphological differences. In turn, this research aims to contribute to the overall knowledge of the impact of radionuclides on the biosphere while specifically unveiling the effects of ionising radiation on a non-model organism found in the contaminated areas of Chornobyl and Fukushima.

To answer whether chronic exposure to radionuclides influences the gene expression of methylation enzymes and sirtuins, a laboratory experiment was set up to expose young pine trees (*Pinus sylvestris*) chronically to enhanced radiation in a controlled environment. This species of pine is native to Chornobyl and exceptionally closely related to *P. densiflora*, a Japanese native pine species commonly found in the FEZ (42). With this experimental setup, we hypothesise that trees chronically exposed to ionising radiation for ten weeks will see an increase in the expression of methylation genes and sirtuins because of their involvement in the protection against radiation-induced DNA damage through chromatin remodelling. We expect to observe differences in the morphology of pines grown under permanent gamma radiation stress. Lastly, we expect differences in chromatin condensation between samples from the control group and the exposure group. However, in this research, we will not investigate differences in chromatin condensation but instead attempt to optimise a working protocol for a flow cytometric approach to analyse chromatin condensation in pine tree samples. This research was conducted as a pilot study to optimise methods and investigate relative expression levels of methylation genes and sirtuins before analyses are conducted on field samples.

EXPERIMENTAL PROCEDURES

Pine tree cultivation and sampling

Seeds of the species *Pinus sylvestris* were imbibed overnight in distilled water, sterilised with 0.5% NaOCl for one minute, and then rinsed thrice with distilled water. The seeds were stratified in the dark at 4°C for one week. The sterilised seeds were sown and seedlings were allowed to grow in a hydroculture for ten weeks on a 14h/10h day/night cycle. The temperature and relative humidity in the growth chamber were kept at 24°C/20°C and 22%/29% respectively. Light was supplied by Valoya LED Grow Lights emitting a wave spectrum from 380 to 780 nm, and seedlings experienced an average PAR density of 138.38 µmol/m^{2}s. For the first four weeks, seedlings were supplied with a medium of distilled water. After four weeks, the water was swapped for a half-strength solution of Hoagland medium (43). Approximately 100 plants were grown under constant radiation from an external cesium-137 source emitting gamma rays at an average dose rate of 682 ± 74 µGy/h, while 100 others served as a control group that was only exposed to the normal background radiation levels.

Twenty samples were taken per condition at the 2, 4, 6, 8, and 10 week time marks. The samples consisted of the root tissue, shoot tissue, and for the 6, 8, and 10 week harvest the shoot tissue was split up into young needles with the apex and the six oldest needles. Buds and emerged side branches of the shoot were also counted. An emerged side branch is considered when the length is greater than 2 mm. Samples were immediately snap-frozen in liquid nitrogen after denoting the fresh weight of the root, stem, and shoot. Pictures were taken prior to snap freezing to determine the length of the roots, stem and shoot using ImageJ (44).

Microscopic imaging of the apical meristems

During the 8th week harvest, the root apex was collected from 5 plants per condition. At 10 weeks, the shoot apex was also collected from 5 plants per group. Shoot tissues were washed in 1% (v/v) Triton X-100 and rinsed with distilled water before fixation. The root and shoot apices were fixed in a solution of 25% (w/v) glutaraldehyde and 0.114 M cacodylate and were left to incubate overnight at 4°C. The next day, samples were dehydrated in an ethanol series, after which they were kept on xylene over the weekend. Next, the samples were embedded in paraffin wax and cut into 7 µm thick sections using a microtome. Sections were deparaffinised and finally coloured with toluidine blue (0.1% v/v). Pictures were taken using a bright field microscope (MC170 HD, Leica).

RT-qPCR

To analyse the expression of genes, a reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was performed on the following genes: SRT1, SRT2, MET1, CMT3, ROS1, and DRM2. In order to design primers for the RT-qPCR analysis, the protein sequences of these genes were obtained from the model organism *Arabidopsis thaliana* from the TAIR database (https://www.arabidopsis.org/). A protein-BLAST with the obtained sequences was performed against a reference database of translated peptide sequences from *P. sylvestris* (45, 46). Next, the homologous cDNA sequence was extracted from the corresponding *P. sylvestris* transcriptome database and used for a primer-BLAST. Primer specificity was ensured by using the pine transcriptome as a custom database for the primer-BLAST, and good primer pairs were selected based on predefined criteria (Table 1, Suppl. A).

Table 1 Genes of interest (GOI) with their selected forward (Fw) and reverse (Rv) primers (from 5' to 3').
[1]Source: Blagojevic (2019) (47)

GOI	Fw	Rv
MET1	AACCCATTTTGCTTCC TTCGTG	GGGAAAGAGATTGCG GAACA
ROS1	CTCGTCCAAGGAAAT CAATCATAGC	AAGGGAGCACATTCA GAGGAT
DRM2	TCGAAGCACCTTTTGA ACGC	CACAAGAGATGGGCT GGAGG
SRT1	TGAAGGCTGGTTCCC AAACA	TTGCGGGGGTAAACT CAAGG
SRT2	TGGTTCAGAGGCAAG TTTTGG	AACACCACCACATTGC TCAC
CMT3	GCCATTGATTCCTGAT TATGCG	CAGTTGGCACAGTTTC ATCCC
ACT[1]	TGACATGGAGAAGAT TTGGC	CATACATAGCAGGCA CATTG
PEX4	AGCTTTCCTGCGATGA CACA	GTCACAAAGCGCACTT GAGG
YLS8	AGAATGGCCTGATCC ACTGC	AGGATCGAGCGAGGC AAAAA

Samples were ground to a fine powder with glass beads using a Mixer Mill MM 400 (Retsch®) prior to treatment with the RNeasy® Mini Kit (QIAGEN). RNA was extracted according to the manufacturer's instructions with slight alterations; approximately 25 mg of PVP-40 (polyvinyl-pyrrolidone, molecular weight: 40 000 g/mol) was added to the shredded frozen samples. A volume of 900 µL of lysis buffer was used instead of 450 µL. A vacuum pump was used for passing the buffers through the column instead of a centrifuge. The purity and yield of RNA were measured using a NanoDrop™ spectrophotometer (ND-1000, Thermo Scientific). Next, the RNA was treated with the TURBO™ DNA-free kit (Ambion) following the manufacturer's instructions, but using 1 µL of buffer, 0.25 µL of DNase, and 1 µg of RNA diluted with RNase-free water to obtain a total reaction volume of 10 µL.

After DNase inactivation, 7 µL of DNase-treated RNA was recovered from the tube and utilized in a 10 µL reverse transcription reaction with the PrimeScript™ RT reagent kit (TaKaRa), following the manufacturer's instructions, using a Doppio thermocycler (VWR). The resulting cDNA was diluted five-fold for further use in qPCR reactions. The remaining volume of DNase-treated RNA was used as a no-reverse transcription control. The RT-qPCR reactions were run in duplicate for the genes of interest (GOIs) in a 96-well plate, but not for the reference genes ACT, PEX4, and YLS8. One no template control and one no reverse transcription control was loaded per GOI. Each reaction contained 5 µL of Fast SYBR Green Master Mix (QIAGEN), 2.5 µL of diluted cDNA solution, 0.3 µL of 10µM reverse primer, 0.3 µL of 10 µM forward primer, and 1.9 µL of RNase-free water for a total reaction volume of 10 µL. The RT-qPCR reaction was done in a qTower³ G (Analytik Jena) using the following cycle settings: 2 minutes at 95 °C are followed by 40 cycles of 20 seconds at 60 °C and 15 seconds at 95 °C. PCR amplification was followed by a melting curve analysis. Due to damage to the qTower³ G, samples collected at weeks 8 and 10 were analysed using QuantStudio 3 (Applied Biosystems) at Hasselt University with identical thermocycling conditions. The same reaction mixes were used but with 1.85 µL of RNase-free water and 0.05 µL of a passive dye called ROX. Ct values were calculated in the qPCRsoft software, with the threshold set automatically, but separately for each GOI. The reference genes ACT, PEX4, and YLS8 were used for normalisation when calculating the relative expression values. These genes were empirically determined to have the most stable expression under various conditions in previous experiments (pers. comm. Brix De Rouck) (48). After averaging the technical replicates, relative gene expression values were calculated using the $2^{-\Delta\Delta Ct}$ method (49).

Nuclei extraction and staining

First, three nuclei extraction buffers were prepared to be tested on pine tree tissue. A woody plant buffer (WPB) was prepared containing 0.2 M Tris-HCl, 4 mM $MgCl_2 \cdot 6 H_2O$, 2 mM EDTA $Na_2 \cdot 2 H_2O$, 86 mM NaCl, 10 mM $Na_2S_2O_5$, 1% (w/v) PVP-25, and 1% (v/v) Triton X-100 (50). Next, an AE buffer was prepared containing 10 mM $MgSO_4$, 50 mM KCl, 5 mM HEPES, 1% (v/v) Triton X-100 and 6.5 mM DTT (51). Finally, Galbraith's buffer was prepared containing 45 mM $MgCl_2$, 30 mM sodium citrate, 20 mM MOPS and 1% (v/v) Triton X-100 (52). Roots and shoots were chopped on ice under various conditions (see Results) and the volumes were filtered using a 50 µm Celltrics filter (Partec). The plant extracts were stained for ten minutes using 1% (v/v) of two different dyes: SYBR Green (excitation/emission wavelengths: 497 nm/520 nm) and DAPI (excitation/emission wavelengths: 358 nm/461 nm). The SYBR Green I stock solution (Invitrogen™, Thermo Fischer Scientific) in DMSO (10,000× concentrate) was diluted 100× and used in a final 10× dilution of 1 µL SYBR Green and 9 µL sample for staining (53).

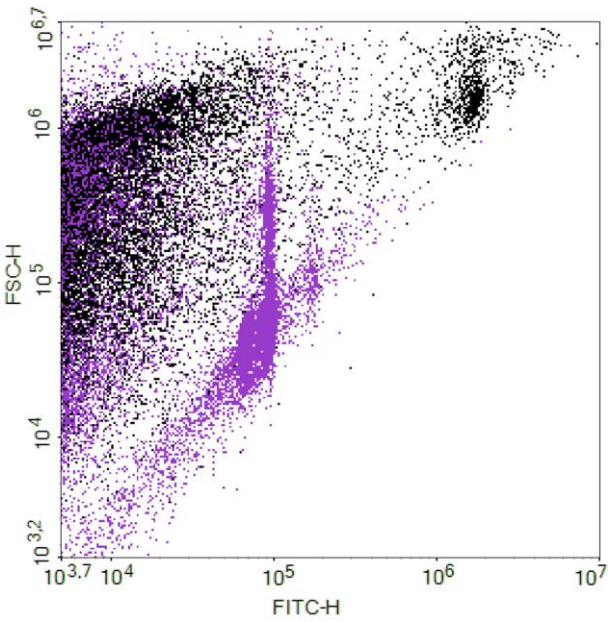

Figure 1 Flow cytometric plot visualising fluorescent responsive events (on a log scale) counted by the NovoCyte Quanteon flow cytometer. FSC-H: forward scatter (hight), measure for the size of counted events; FITC-H: fluorescein isothiocyanate (height), measure for the SYBR Green fluorescent response of events; red: counted events in an extract of *Lemna minor* using Galbraith's buffer; black: counted events in an extract of 14 week old *Pinus sylvestris* roots using Galbraith's buffer.

Next, 20 µL of unstained and stained extracts were analysed using a NovoCyte Quanteon flow cytometer (Agilent). The forward scatter (FSC) and side scatter (SSC) thresholds were initially set at 1 000 for both. Approximately 80 mg of *Lemna minor* was chopped for 2 min, stained with SYBR Green and analysed using the flow cytometer as well. After optimising the chopping procedure, we compared the WPB, AE buffer and Galbraith's buffer for their efficacy to isolate nuclei for flow cytometric analysis. To confirm the extraction was successful, the stained nuclei were visualised using a fluorescence microscope (Eclipse TI, Nikon, excitation: 470 nm, emission: 535 ± 20 nm).

Statistics in R

The phenotypical data was log-transformed and analysed using 2-way ANOVA with condition and time of harvest as factors. A t-test was performed to compare the control and irradiated groups per timepoint. The Wilcoxon-rank sum test was performed in case normality could not be assumed. The emergence and bud count of irradiated and control pines were compared using a χ^2-test. The control and irradiated groups of the relative gene expression analysis were compared using the Wilcoxon-rank sum test.

RESULTS

Flow cytometry try-outs

Before the three extraction buffers were compared, the chopping method was optimised with WPB as the standard buffer. First, shoot and root samples were chopped in 1 mL and 2 mL of WPB for approximately 1 min, 2 min and 3 min. It was found that no more than 100 mg of tissue should be chopped but ideally, 80 mg of tissue should suffice. The chopping time should also be as minimal as possible because pine needles that were purposefully overchopped for over four minutes yielded cloudy extracts that precipitated quickly (54). Chopping for one minute was found to be sufficient to extract nuclei from the roughly chopped plant material. Increasing the standard 1 mL volume of the extraction buffer did not seem to yield better extractions. The WPB was modified by adding 0.5% (w/v) β-mercaptoethanol and 5% (w/v) PVP-40 (55), but those additions did not improve the nuclei extract purity; a cloud of low fluorescent debris remained present at a FITC intensity of 10^4 and lower. A 10 µm Celltrics filter

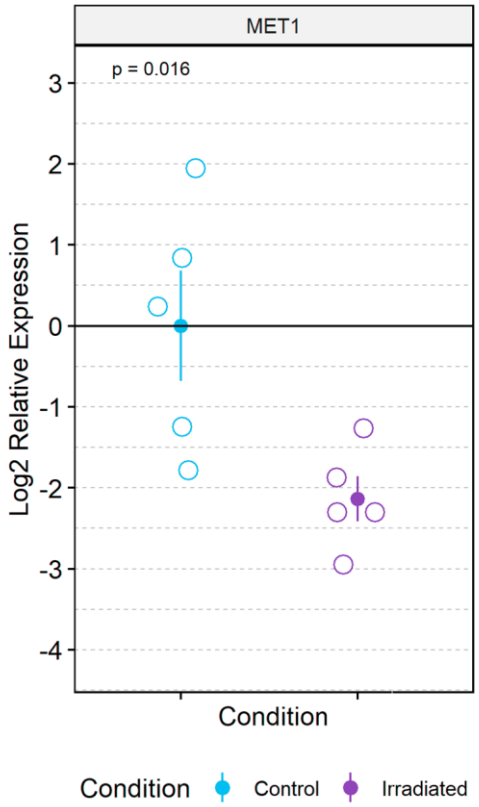

Figure 2 Relative expression levels of MET1 in the root tissue of plants harvested in the 10th week of the cultivation (n = 5 per condition). Calculations for the $2^{-\Delta\Delta Ct}$ method were performed in R. Error bars indicate the variation within each population of 5 biological variates. The p-value was calculated using the Wilcoxon-rank sum test.

instead of the 50 µm was also tested but was quickly discarded because we noticed a decrease in the number of events using the 10 µm Celltrics filter. The concentration of SYBR Green was used as described by Clarindo et al. (53), and therefore not altered during our optimisation try-outs. Nuclei were stained for 10 min, 30 min and 60 min, but the time of the staining seemed to not have any effect. Pelleting the nuclei at 250g, 1000g, and the max speed of the centrifuge for 5 min was also tested. The supernatant was removed and analysed along with the resuspended pellet. The supernatant seemed to not contain many events, while more events were counted in the pellets at all three speeds. Pelleting at 250g seemed to increase the purity of the extract after resuspending the pelleted nuclei, but pelleting at 1000g and max speed seemed to include more impurities. Although pelleting at 250g yielded better results, the pellet was relatively weak and easily disturbed, while the 1000g and max speed pellet were firmer and less easily disturbed. It remained a challenge to pellet consistently at the low speed of 250g, resulting in an unreliable purification step. The gain of the side scatter channel (SSC) and fluorescent signal (FITC) were both lowered from 400 and 543 respectively to 50, revealing a few more events higher than the earlier FITC limit of 10^7, although these events comprised less than 1% of the total number of events. Therefore, changing the gain may yield a more complete view of the events without a cut-off point, but it does not yield better results.
Because flow cytometry seemed to not generate usable data, we resorted to fluorescence microscopy to visualise the extracted nuclei using the WPB, AE buffer and Galbraith's buffer. To investigate whether pelleting at higher speeds (1000g and max) yields more nuclei, we split the extract into two equal volumes and pelleted one aliquot at 250g and 1000g as done before for the flow cytometric analysis. Most nuclei were visualised using the WPB extract (Suppl. G). Pelleting at 250g resulted in more stained nuclei than pelleting at 1000g, where almost no nuclei were stained and only debris remained stained. However, whether pelleting at 250g yield a higher count of stained nuclei remains unclear because similar quantities of fluorescent material were observed in the 250g pelleted and non-pelleted extracts. The nuclei extractions using the AE buffer and Galbraith's buffer contained very few fluorescently stained nuclei. In the case of the AE buffer, the extract was cloudy and precipitated within half an hour.

Due to remaining difficulties in visualizing the nuclei among other events counted by the flow cytometer, a positive control of *Lemna minor* was used as a reference measurement (Figure 1). The red events around a fluorescent FITC-signal of 10^5 and FSC-signal between 10^4 and 10^5 are potentially SYBR Green stained nuclei extracted from *Lemna minor*. The black events clustered together around a FITC-signal of 10^6 are potentially SYBR Green stained nuclei as well extracted from *Pinus sylvestris*. Even with *L. minor* as a positive control, which generated clearer clouds of events we believed to be nuclei, and all of the above conditions tested, still, no reliable data could be

generated from pine tree samples using the NovoCyte Quanteon flow cytometer. Therefore, we were not able to use flow cytometry to determine which extraction buffer has the highest efficacy.

Because we were unable to generate data from the pine tree samples using flow cytometry, even though we had visually confirmed our extraction method works, we cannot determine which extraction buffer performs best. An extraction and staining procedure was tested and improved by excluding certain changes to parameters in the sample preparation. An actual working protocol for visualising stained nuclei using flow cytometry could not be established. From what little data we could gather from the flow cytometric approach, we found that the WPB yielded the highest count of fluorescent events, followed by the AE buffer and Galbraith's buffer. We also found that the extract using Galbraith's buffer to extract nuclei yielded a cluster of events around a fluorescent signal of 10^6 (Figure 1), which was not present for extracts using WPB or AE buffer. Finally, for the nuclei extracted from *Lemna minor*, we were able to generate a cell cycle plot (Suppl. H).

Relative expression of methylation enzymes

The expression of all six GOIs remained unaltered in the shoots and old needles of irradiated pine trees compared to the control group. At each timepoint (weeks 2, 4, 6, 8, and 10), no significant differences were found in the expression of any GOI in the needles. However, in the roots, the expression of MET1 is significantly lower in irradiated plants compared to control plants (p=0.016) (Figure 2). Trees grown under permanent gamma irradiation have a log two fold lower expression of MET1 in the roots at week 10, but also a borderline insignificant lower expression of SRT2 (Suppl. E). Such borderline insignificant lower expression was also found for MET1 and SRT2 in the old needles at 10 weeks (Suppl. E). Interestingly, no such (borderline) significant results can be found in any tissue at other timepoints, except for a borderline insignificant difference in MET1 and SRT1 expression in the old needles of 6-week-old pine trees (Suppl. F). Most notable is that the MET1 and SRT1 expression is upregulated in irradiated trees at week 6, while MET1 expression is significantly less expressed in the roots of 10-week-old pines. Although borderline insignificant, the expression of

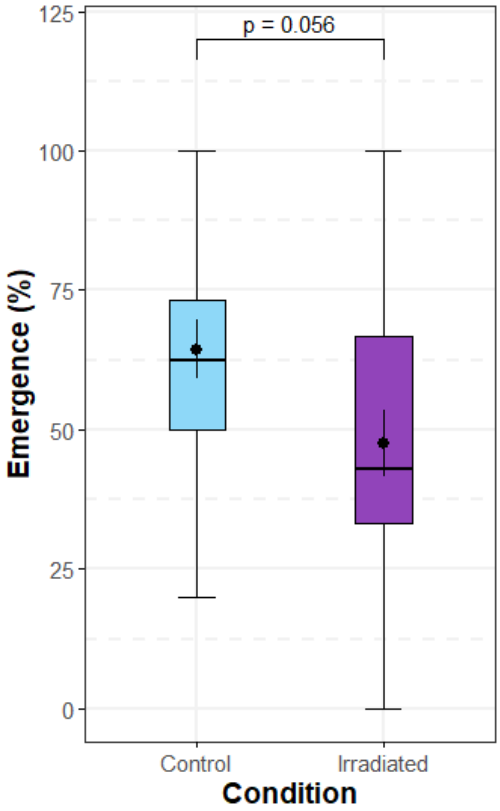

Figure 3 Boxplot of the emergence percentage (%) of buds that emerged as side branches of the shoot at week 10. Groups were compared using a χ^2-test (control mean: 63.04%; irradiated mean: 47.67%).

MET1 in the old needles and shoot at week 10 is still downregulated, contrasting with the slight upregulation in old needles at week 6. Regardless of significance, all GOIs in all three tissues of 10-week-old irradiated pine trees seem to experience a downregulation compared to the control pine trees.

Phenotyping

The length and weight of all tissues (root, shoot, and stem) and their total were compared between the control and irradiated groups after log transformation (Table 2). Normality could not be assumed for the parameter root length, thus a Wilcoxon rank-sum test was used to compare the mean root lengths per timepoint. An ANOVA comparison was also performed per parameter. A significant effect of the radiation (p=0.0112), growth period ($p=<2^{-16}$) and their interaction (p=0.0211) is observed for the total length of plants. While the effect of the week is omnipresent for all parameters, the significant effect of the condition and the interaction term is specific to the total

length and root length (radiation: p=0.0255; radiation-growth period interaction: p=0.00213). The overall total weight of the plants is not significantly influenced by radiation or the interaction term. This is also the case for all tissues separately that contribute to the total weight. Moreover, the same significance differences as observed for the total length are seen in the root length, but not in the length of any other tissue.
The change in total length and weight was plotted for the entire growth period of ten weeks (Figure 4). A significant difference occurs in the 4th and 6th week in the total length of plants between the control and irradiated group. The effect is more significant at week 6 than a week 4. Such effect is not observed for the total weight, however, a significant difference between the control and irradiated groups was observed in the 10th week. Notably, the total length and weight of control plants are at no point lower than those of the irradiated plants. Similar graphs were constructed for the tissue-specific lengths and weights, but not for tissues with no significant differences between control and irradiated plants (Suppl. B-D). As for the total length, the same significant difference between the control and irradiated groups was observed in the root length (Table 2, Suppl. B), although the significant difference in the 6th week appears lower. The same significance level as observed for the total weight in the 10th week is found in the stem and root weight at week 10 (Suppl. C and D).

Lastly, the frequency of buds and branches was compared between the control and irradiated group. The ratio of branches to the number of buds was used as a measure for the emergence of side branches of the shoot (Figure 3). The control group had an average emergence of 63.04%, while the irradiated group had an average emergence of 47.67%. A χ^2-test revealed no significant difference in the emergence of side branches between the two conditions at week 10. The same statistical test was used to compare the counts of buds (including both emerged and non-emerged buds) at weeks 6 and 8, revealing a significant difference in the total number of buds at week 6 (Suppl. I).

Root and shoot apex visualisation
From the five control and irradiated roots, the two best ones were selected (Figure 5). The same was done for the shoot apex samples (Figure 6). Visually, the apical root meristems of the control and irradiated samples look alike. No major growth deficits or morphological abnormalities are visible. However, the apical meristem located in the shoot is visibly different (Figure 6). Most notable, is the lack of leaf primordia in the irradiated apex compared to the clear bulges near the apex of the control group. Leaf primordia seemed obscured or absent from all four replicates in the irradiated group, while they were clearly present in the three replicates of the control group. Secondly, the apical part of the meristem of the irradiated plant (Figure 6.B) is more globular compared to the control apex (Figure 6.A). This appears to be the case for all replicates.

DISCUSSION
Extracting and visualising nuclei
First, it should be noted that for no approach of the numerous attempts to visualise pine tree nuclei using flow cytometry, clear clusters of nuclei containing 2n, 4n or more genetic material were visualised. Normally, different clouds of different ploidy levels should be visible since the extracted nuclei usually originate from a heterogeneous population of nuclei at all stages of the cell cycle and various levels of polyploidy. However, this was not the case for the pine tree extracts we prepared. In Figure 1, nuclei from pine roots were extracted using Galbraith's buffer and possibly one cluster of nuclei in the G1- and G2-phase was visualised. However, any other clusters of higher ploidy levels are absent. A possible explanation for this could be that the roots of pines are mitotically inactive besides the meristem in the tap root and local meristems in the root hairs (56). Another possibility is that the suspected cloud of nuclei is only autofluorescent debris. This is rather unlikely given the position of the cluster separately from most other events. For the extracts of *Lemna minor*, however, a cell cycle plot was constructed based on one cloud of events we assumed are nuclei (Suppl. H). Higher phases of polyploidy were not detected because *L. minor* does not endoreduplicate in de absence of an external stressor (57, 58), hence only nuclei in the G1- and G2-phases were detected.

Because pine trees have a large genome (between 18 000 and 40 000 Mbp) compared to other plants like *L. minor*, we expect their nuclei to be bigger as

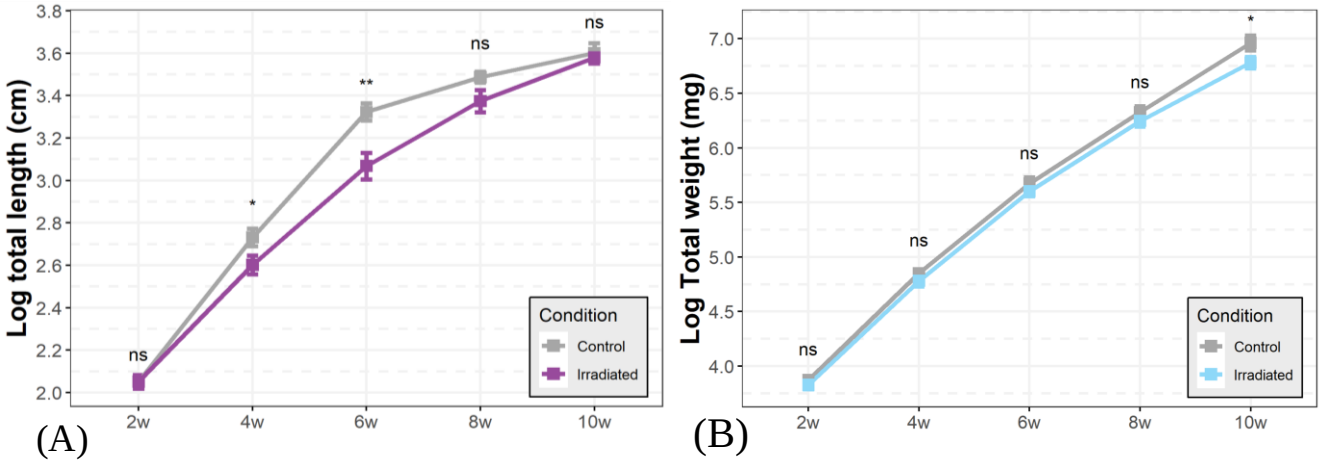

Figure 4 (A) Line plot of the log total length (in cm) and **(B)** log total weight (in mg) of plants harvested at time points 2, 4, 6, 8 and 10 weeks. A t-test was performed to compare the irradiated group with the control group at each time point. Specific p-values can be found in Table 1. (Significance codes: 0 < p < 0.001 ***; 0.001 < p < 0.01 **; 0.01 < p < 0.05 *)

well (59). This should result in a higher FSC-signal because the FSC is a measure of the size of events. An increase in fluorescence intensity (FITC-signal) is logical as well because a larger genome should be stained more and thus fluoresce more intensely. Therefore, we expect the cloud of events clustered around a FITC-signal of 10^6 to be actual pine tree nuclei (Figure 1). Although, more experiments are needed to confirm whether the cluster around the FITC-signal of 10^6 is actually a group of nuclei. The use of different dyes like propidium iodide (PI), DAPI, or other dyes used for nuclei staining might prove beneficial for visualising nuclei using flow cytometry in future experiments.

In contrast to the cell cycle plot of L*emna minor* (Suppl. H), all data fitting failed when the cloud of suspected nuclei of *Pinus sylvestris* was fitted on the cell cycle plot. One possibility is that the G2-phase is absent from the root extract (56). It is also possible that the cluster does not contain nuclei, or that the extract contains too few nuclei to be differentiated among the debris by the flow cytometer. The observed cluster of events could be fragments of damaged nuclei or other forms of genetic material (for example, chloroplast and mitochondrial DNA) that have aggregated together. Another possibility is that the extraction buffers isolate nuclei successfully, but that they also extract tannic acid, polyphenols or other metabolites that may interfere with the staining procedure of nuclei or measurements in the flow cytometer (55). To counter these possible interfering molecules, we modified the WPB by adding β-mercaptoethanol and PVP-40, but to no avail (55). These modifications did not yield better results compared to the use of the unaltered WPB, suggesting an unknown factor is at play, causing interference in the flow cytometric analysis of pine tissue samples. While the modification of the WPB did not improve nuclei isolation, pelleting at 250g seemed to increase the purity of the nuclei extracts. However, pelleting at higher speeds (1 000g, max) was found to disrupt the nuclei, only leaving behind nuclear debris that was faintly stained during fluorescence microscopy, while the resuspended 250g pellet and unpelleted extracts yielded more stained intact nuclei. It was also found that the chopping time should be kept low to not overchop the samples like we purposefully did to investigate the chopping time. This was also suggested by Loureiro et al., who found that overchopping could lead to unsuccessful flow cytometry runs (54). In contrast to their findings, we found the WPB unsuccessful in isolating nuclei for flow cytometric analysis from problematic tissue such as pine needles. Given the non-model nature of pines and the presence of interfering molecules such as polyphenols, we thought the WPB to be well suited for our samples. Even more so, Marum et al. have successfully extracted and stained nuclei for flow cytometry from *in vitro*-grown pine needles using WPB, further validating the suitability of WPB for flow cytometry analysis (60). Contrary to our belief, we found that the WPB did not perform better than the AE buffer or Galbraith's buffer. Interestingly, fluorescence microscopy and flow cytometry results do not confirm the same thing. From

fluorescence microscopy, it appeared that the WPB was the best-suited extraction buffer as we were not able to spot as many stained nuclei in the other two buffer extracts (Suppl. G). In contrast, flow cytometry revealed a small cloud of what we think are nuclei (Figure 1) in the AE buffer and Galbraith's buffer, but not in the extracts using the WPB. These inconclusive results indicate a lot remains unclear about the choice of extraction buffer and further experiments are needed to define a final answer. As for the flow cytometry approach, different dyes for the staining of nuclei may help bring clarity in future experiments. Combining different dyes may also help visualise nuclei alone, excluding incidentally stained debris or damaged nucleus fragments. Preparing extracts from pine trees cultivated in a similar *in vitro* setup as used by Marum et al. may also yield different results compared to extracts from hydroculture pines (60). We assume our extraction method for extracting nuclei works on *L. minor* because we were able to construct a cell cycle plot. By extension, we also believe our extraction method works on *P. sylvestris* as we visualised stained nuclei using fluorescence microscopy (Suppl. G) and we believe a cluster of nuclei was visualised around a FITC-signal of 10^6 (Figure 1). Besides the first steps presented here towards an extraction procedure for pine tree nuclei from needles and roots, more research is needed to reveal what exact interference occurs that obscures flow cytometry data. Perhaps better-suited methods for extracting nuclei exist that we have not been able to include in our flow cytometry tests. Comparing different tissues of pine seedlings, such as the buds, meristems, oldest needles, roots, stem, youngest needles, etc. may allow the identification of the most problematic tissue containing the most cytosolic compounds for flow cytometry. Because of the low mass of buds or the meristem, samples might need to be pooled from different individuals, which might also prove beneficial for larger tissues like needles to eliminate the effects of biological variation. Perhaps the procedure to remove tannins from medicinal plant extracts by Gong et al. might be adapted to remove phenolic compounds from pine tree extracts (61).

Disturbance of apical dominance

First, it was found that although there is a difference of 15.37% in the total emergence of side branches at week 10, irradiated pines experience a barely insignificantly lower emergence of side branches (Figure 3). At week 6, a significant difference was noticed in the distribution of the number of buds (emerged and non-emerged) between the control and irradiated groups ($p = 0.01725$) (Suppl. I). The population of control plants contains more individuals that have only one bud, while the population of irradiated plants contains more individuals with zero buds. This difference appears significant, while at week 8 no such difference was found. Taken together, it can be stated that exposure to gamma radiation might influence the outgrowth of side branches of the shoot, although not at all timepoints. As earlier research has demonstrated that ionising radiation is capable of disturbing apical dominance regulators like auxin and cytokinin, we expected to observe a significant difference between the number of buds in the control and irradiated populations, but also a difference in the emergence of side branches (7, 15). When the apical shoot meristem is damaged following radiation exposure, buds below the apex assume dominance and become the new main branch (62, 63). Because we noticed a similar percentage of emergence in the irradiated trees as in the control trees, it is possible the auxin pathway was not heavily disturbed in plants exposed to ionising radiation for 10 weeks. This is further supported by the fact that we never observed morphological abnormalities in the apical dominance of plants during harvest. No other deformities were visualised in the apical shoot meristem, except for the apparent absence of the leaf primordia and the globular shape of the meristem of irradiated samples (Figure 6.B). One possible explanation for this is that the dose rate of 682 µGy/h was enough to exhibit a local effect on the meristem resulting in a globular shape of the meristem, and by inhibiting or slowing down the development of leaf primordia. In line with this, the dose rate of 682 µGy/h may not be enough to induce larger-scale changes like the emergence of buds into side branches. Another possibility is that none of the four irradiated meristems are correctly prepared slides. Although unlikely, the meristem, leaf primordia and other apparent structures might be missing from these pictures because they are situated in another plane from which the slides were made.

Table 2 Summary of the means (± S.D.) of the control group and irradiated group per timepoint per parameter (length (in cm) and weight (in mg) in total and per tissue root, shoot, and stem). T-tests were performed per week to compare the control and irradiated groups. All data were log-transformed (except for root length).[1] (Significance codes: 0 < p < 0.001 ***; 0.001 < p < 0.01 **; 0.01 < p < 0.05 *)

[1]Normality could not be assumed, so the Wilcoxon-rank sum test was performed on the untransformed raw data as an alternative to the t-test.

Parameter	Week	Control	Irradiated	p-value
Total length (log)	2	2.051 ± 0.14	2.051 ± 0.14	0.9983
	4	2.731 ± 0.19	2.602 ± 0.20	0.04086 *
	6	3.323 ± 0.18	3.068 ± 0.28	0.001533 **
	8	3.487 ± 0.11	3.374 ± 0.23	0.06178
	10	3.600 ± 0.21	3.580 ± 0.14	0.6923
Total weight (log)	2	3.872 ± 0.16	3.828 ± 0.17	0.4122
	4	4.851 ± 0.16	4.783 ± 0.23	0.2829
	6	5.674 ± 0.25	5.603 ± 0.20	0.3292
	8	6.327 ± 0.24	6.247 ± 0.24	0.2983
	10	6.963 ± 0.30	6.782 ± 0.26	0.0391 *
Stem length (log)	2	1.276 ± 0.17	1.251 ± 0.11	0.5868
	4	1.251 ± 0.16	1.326 ± 0.14	0.6791
	6	1.326 ± 0.14	1.307 ± 0.15	0.5635
	8	1.307 ± 0.16	1.327 ± 0.13	0.6549
	10	1.327 ± 0.17	1.354 ± 0.13	0.04165 *
Stem weight (log)	2	2.870 ± 0.20	2.868 ± 0.21	0.9787
	4	2.894 ± 0.22	2.957 ± 0.20	0.3479
	6	2.982 ± 0.26	3.041 ± 0.25	0.4742
	8	3.214 ± 0.23	3.136 ± 0.29	0.3475
	10	3.805 ± 0.31	3.677 ± 0.24	0.1305
Shoot length (log)	2	0.711 ± 0.16	0.677 ± 0.17	0.5227
	4	0.988 ± 0.10	0.982 ± 0.10	0.8845
	6	1.556 ± 0.12	1.483 ± 0.12	0.05507
	8	1.712 ± 0.12	1.648 ± 0.15	0.1497
	10	1.864 ± 0.15	1.802 ± 0.15	0.1237
Shoot weight (log)	2	3.120 ± 0.22	3.052 ± 0.21	0.9787
	4	4.141 ± 0.19	4.125 ± 0.20	0.3479
	6	5.104 ± 0.23	5.005 ± 0.19	0.4742
	8	5.763 ± 0.23	5.638 ± 0.18	0.3472
	10	6.366 ± 0.28	6.207 ± 0.24	0.1305
Root length[1]	2	2.159 ± 0.75	2.343 ± 0.64	0.279
	4	9.093 ± 2.81	7.344 ± 2.73	0.0375 *
	6	19.611 ± 4.85	13.931 ± 5.97	0.0143 *
	8	13.931 ± 3.47	21.218 ± 5.88	0.133
	10	23.929 ± 6.43	26.792 ± 4.75	0.622
Root weight (log)	2	1.966 ± 0.34	1.908 ± 0.30	0.3257
	4	3.830 ± 0.20	3.624 ± 0.35	0.8008
	6	4.659 ± 0.31	4.572 ± 0.39	0.1461
	8	5.353 ± 0.34	5.333 ± 0.36	0.06747
	10	6.044 ± 0.38	5.837 ± 0.33	0.04893 *

While we found some deformities in the shoot meristems of pines exposed to a dose rate of 682 µGy/h (Figure 6), Blagojevic (2019) found major deformities only at higher dose rates starting from 1 mGy/h, up to 540 mGy/h (47). At the lowest dose rates of 1 mGy/h, leaf primordia were visibly larger than the meristem, but at higher dose rates of 100 mGy/h and more, major deformities occurred rendering the meristem unrecognisable. Such extreme effects are absent at the dose rate of 682 µGy/h. Curiously, we could not recognise leaf primordia that have grown visually larger than the meristem in irradiated trees as Blagojevic observed (47). In our case, leaf primordia are completely absent (Figure 6.B), yet no other deformities occur besides this absence and a more globular shape of the irradiated meristem. Note that comparing Figure 6.A and 6.B is a subjective method of evaluating the differences between the control and irradiated groups. No quantitative method was applied to these pictures, as they serve to support the phenotype observations. Taking these limitations into account, we suggest further research is needed; including more replicates may shed more light on the findings presented here.

Generally speaking, pines grown under radiation stress seem to withstand the dose rate of 682 µGy/h rather well. Of note is that this dose rate is three to four orders of magnitude lower than what is regularly used in other plant exposure experiments with for example rice, *Arabidopsis thaliana,* etc. (64, 65). Using a lower dose rate for a longer period of time allows us to omit acute effects and conduct a long-term exposure experiment of ten weeks in the lab that is closer to a field-relevant dose rate than any other plant exposure experiment in the lab in the past. (4, 66). It is also similar to the lowest observable effect dose rate (LOEDR) at which cytogenetic effects occurred in different plant populations inhabiting radiation-contaminated areas (4, 67, 68, 69, 70). To conclude, it may be possible that the relatively low dose rate of 682 µGy/h is not high enough to incur adverse effects related to branch emergence and the loss of apical dominance in 10-week-old Scots pines, but it may be enough to incur local adverse effects such as major meristematic deformities (Figure 6.B).

The root as sensitive tissue

A remarkable trend that becomes evident when looking at Figure 4 is that irradiated plants seem to grow less than control plants. For the total length of plants (Figure 4.A), irradiated seedlings seem to have been able to maintain a steady growth curve, while control plants seemed to gain more height early on, only to slow down their growth after the 6-week mark. This observation suggests that control plants might have had a better opportunity to invest in their growth in a stress-free environment compared to plants experiencing radiation stress. Combined with the ANOVA comparison results, where a clear effect of the interaction of radiation and growth period on the total length (and root length) occurred, it is possible that the growth of seedlings was slowed down after week 6 due to the effect of radiation. The sudden lag in the total length of control plants after week 6

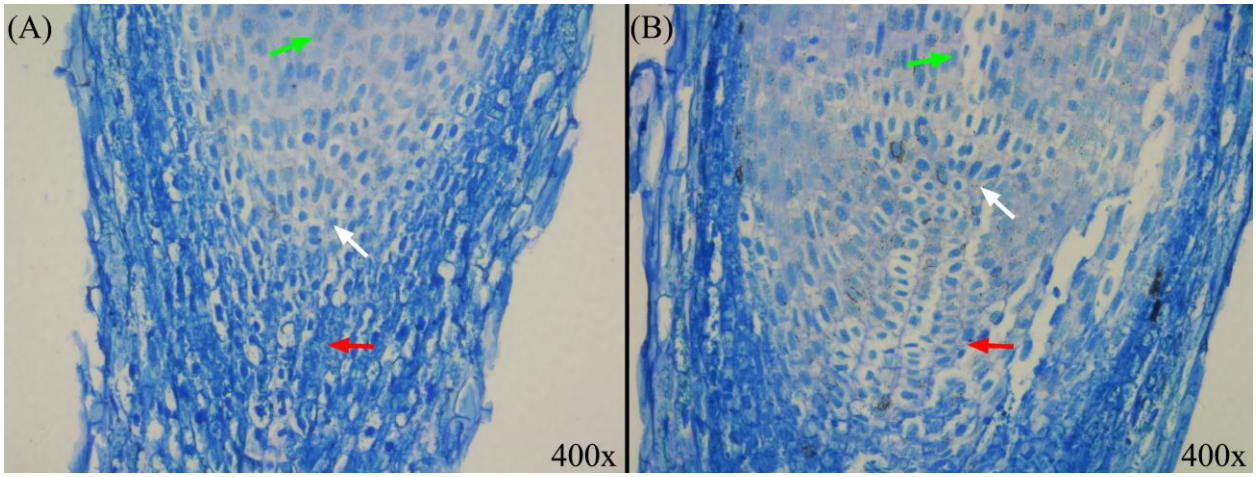

Figure 5 Microscopy visualisation of the toluidine blue stained apical root meristem of a pine tree from the **(A)** control group and **(B)** the irradiated group. Green arrow: stele cells running vertically up the root; white arrow: meristematic zone; red arrow: layers of root cap cells protecting the root meristem. Contrast and brightness were increased using Adobe CC Photoshop 2020.

may be caused by the hydroculture setup that was used to grow the plants in. In this setup, plant roots were closely entangled in the Hoagland solution after week 6, while the roots of irradiated plants were already shorter and therefore less prone to entanglement. The result could be less retardation in root length among irradiated trees. This explanation justifies the lack of significant differences in root length observed at later timepoints. Another possibility is that plants had to compete for nutrients in the Hoagland solution resulting in the observed effect. This effect is also supported when looking at the length of the roots (Suppl. B). The shoot did not display any retardation in length after week 6, suggesting the observed retardation in total length is attributable to the root tissue or stem tissue given the significant difference in length of both these tissues at week 10 (Suppl. B and C). Of note, is that the mean total length of irradiated stems is lower at weeks 8 and 10 than the initial starting length at week 2. Considering the nonsensical nature of the significant difference in average stem lengths at week 10, we can attribute the differences in total length solely to the root tissue. Any significant difference in stem length between the irradiated and non-irradiated groups should be disregarded because these differences can be ascribed to biological variation. That leaves us to conclude that indeed, the differences observed in the total length and weight of plants can be solely attributed to differences in the root length and weight.

Lastly, the results presented here are in accordance with findings by Blagojevic (2019) who found no difference in the total length of seedlings at 12 days, similar to our 2-week timepoint (47). Even at the lowest dose she had used (1 mGy/h), no difference in total plant length was found, as is the case for our results. Interestingly, in terms of total plant weight, control plants exhibit the trend to outgrow the irradiated plants slightly more as time goes on because of the increasing distance between both line plots. This trend yields a significantly lower biomass of irradiated plants at the 10-week harvest time, suggesting that as time goes on, plants grown in a stress-free environment are able to generate

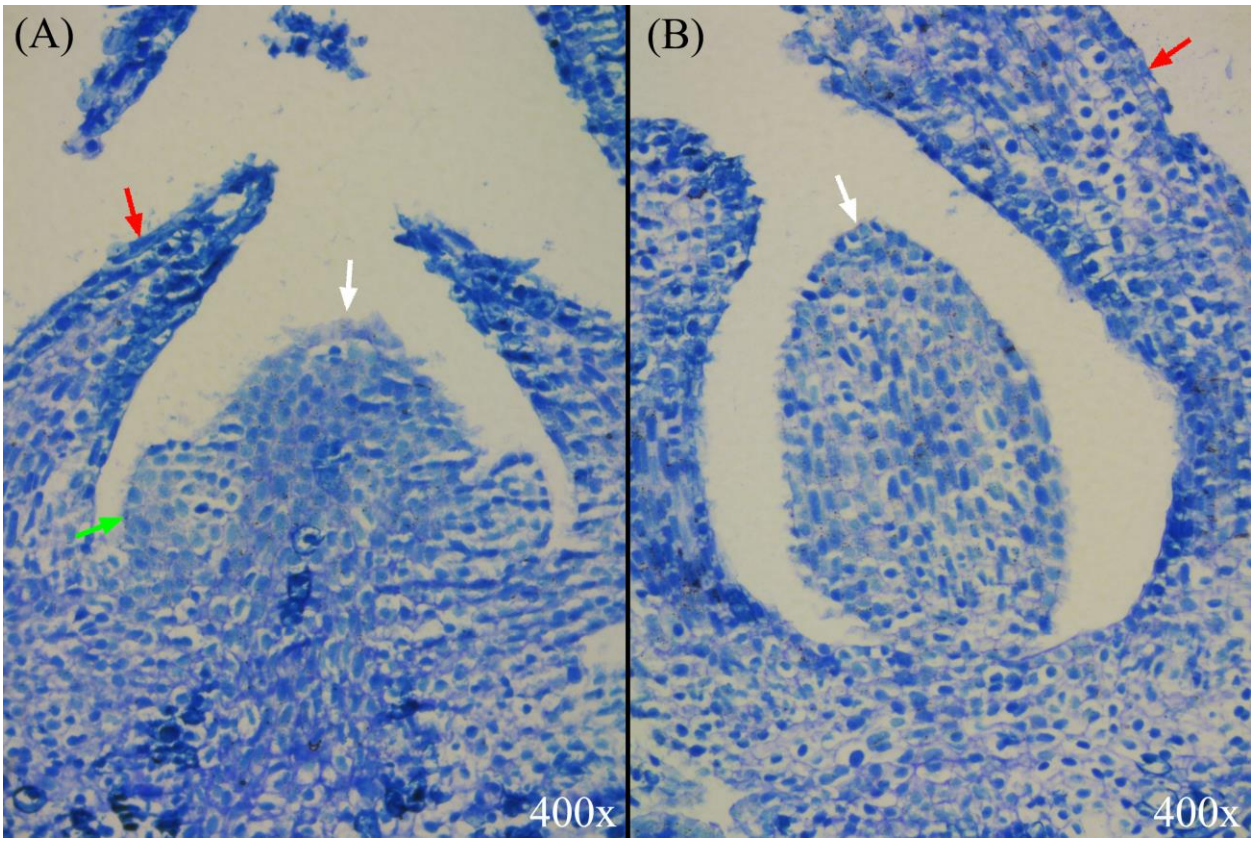

Figure 6 Microscopy visualisation of the toluidine blue stained apical shoot meristem of a pine tree from the **(A)** control group and **(B)** the irradiated group. Green arrow: leaf primordium (absent in B); white arrow: meristem; red arrow: young leaf. Contrast and brightness were increased using Adobe CC Photoshop 2020.

more biomass compared to stressed plants. Due to the continuous radiation stress from seed emergence to harvest, irradiated seedlings could have had to invest more energy in their defensive or repair mechanisms causing them to lag behind on overall growth compared to control plants (24).

Similarly to the contribution of the root length to the differences in the mean total length, we can attribute the difference in the total weight at week 10 (Figure 4.B) to the root weight in the same manner (Suppl. D). A significant difference in root weight was found at week 10, which coincides with the difference in total weight at the same timepoint. It can be stated that, like differences in mean total length, the difference in mean total weight is solely attributable to differences in root weight, excluding other tissues from this contribution. This suggests that needles and the stem are not affected by a dose rate of 682 µGy/h, while the roots do exhibit signs of adverse effects. However, water radiolysis occurs in the water – and later Hoagland medium, due to exposure to gamma radiation (71). This chemical process produces a lot of reactive oxygen species (ROS) in the hydroculture exposing the roots to an extra stressor, although previous research has found the effects to be minimal to nihil (71). In contrast, *Lemna minor* and *Arabidopsis thaliana* have both been found to experience ROS as an extra stressor on the roots in a hydroculture setup (pers. comm. Nele Horemans) (72). Other research supports the claim of adverse effects of ROS on plants (73, 74, 75). This means that we are potentially looking at the effects of ROS on roots and not at the effects of ionising radiation on roots, despite Smith & Wiley's claim that ROS has almost no extra effect on plants (71). It must be noted that their experiments did not investigate the production of ROS in a hydroponic setup, meaning the observations of ROS as an extra stressor for *Lemna minor* and *Arabidopsis thaliana* in a hydroculture may as well be applicable to the hydroculture of *Pinus sylvestris*. If differences in root length and weight are the actual result of radiation exposure, the root tissue may possibly be the most sensitive tissue in Scots pines compared to the needles or stem. To confirm this assumption, future research is advised with a different cultivation setup. Perhaps a different experimental setup would yield a more accurate view of the growth differences in future experiments. Future research may also investigate the tendency (although minimally present) of plants grown in a stress-free environment to invest more in their growth compared to stressed plants (Figure 4). Whether this trend continues beyond the 10-week timepoint, might also be interesting as significant differences in root weight only appear at this late harvesting timepoint. Taking into account multiple parameters that help quantify the concept of "growth" might also prove beneficial in future experiments. Growth is a broad term influenced by many different parameters of which the most prominent ones are already given in this research, but other quantifiers for growth such as hormone levels could help clarify the small differences observed here. Other interesting parameters to include when investigating plant growth could be water content by looking at the fresh weight/dry weight-ratio, cell wall structure and its influence on cell elongation and sugar and protein content.

Root meristem resilience

Unlike our finding that roots are phenotypically affected by radiation, we could not confirm this at the microscopic level. No deformities or other morphological abnormalities that would indicate adverse effects of radiation were observed in the root meristem (Figure 5). A similar composition of root tissues was recognisable in both the control and irradiated root meristem, which was not the case for the apical shoot meristem (Figure 6). The absence of signs of adverse effects of radiation in the root tissue is in contradiction to what was found in the macroscopic analysis of the phenotype, but also in contrast to what is found in literature. A review by Geras'kin et al. (2013) highlights previous field studies on Scots pines with adverse effects on the morphology of root meristems and cytogenetic damage at similar or even lower dose rates (4). Cytogenetic alterations in seedling root meristems were observed in Scots pines exposed to 2.5 – 27 µGy/h in the CEZ, and in Scots pines exposed to 0.8 – 14.8 µGy/h in the radioactively contaminated Bryansk Region (4, 76, 77, 78). Even at the higher dose rate of 682 µGy/h, we were not able to confirm the findings by Geras'kin et al. with our results. Possibly, there is a huge discrepancy between field-irradiated and laboratory-irradiated pines. The duration of exposure is a significant factor to take into account. Usually, laboratory cultivations investigate the effects of acute irradiation, because

long-term exposure to gamma radiation in laboratories is more complex and time-consuming than a short acute exposure experiment (79, 80). Although our pines were grown for 10 weeks under constant ionising radiation, and are therefore considered to have experienced "long-term" irradiation, it appears major differences still remain between field and laboratory experiments investigating the effects of ionising radiation on pines. Naturally, field experiments include far more effects of multiple stressors that cannot be controlled (temperature, soil composition, other forms of pollution, etc.), while a laboratory experiment aims at the investigation of only one stressor at a time.
In summary, root meristems appear more resilient to ionising radiation, which is in contrast with previous findings both in literature and our own phenotypical data.

The downregulation of MET1
Due to issues with the ACT measurements, only PEX4 and YLS8 were used as reference genes for CMT3, SRT1, and SRT2 measurements of the shoots at week 8 and the old needles at week 10. For the MET1, ROS1 and DRM2 measurements in shoots at week 10, ACT was left out as well. This resulted in a higher spread in the data at later timepoints compared to earlier timepoints. Especially the control group seems prone to this effect, which could indicate a high biological or technical variation. Ct-values of replicates never differed more from each other than 0.1 – 0.8 Ct-value, aside from a few outliers. Because most Ct-values of replicates are similar to each other, a high technical variation is more plausible than a high biological variation. This leads us to believe that the high spread in data observed at later timepoints is mostly caused by technical variation after switching from the qTower^3 G to the QuantStudio 3. The use of only two reference genes instead of three may also contribute to the higher variation of data at later timepoints (Suppl. E).
Despite this considerable variation in our data, we found a significantly lower expression of MET1 in the root tissue at week 10 (Figure 2). Interestingly, this lower expression of MET1 is not to be found in the root tissue at any other timepoint. Instead, the only similar trend at week 10 is found in the slightly lower expression of MET1 in the old needles, although not significant (Suppl. E). These findings suggest that exposure to ionising radiation lowers the expression of the maintenance methylation enzyme MET1, especially in the root tissue. Surprisingly, this is not commonly found in literature. Instead, it has been reported that pines native to the CEZ are hypermethylated and that the level of hypermethylation is dependent on the level of radiation exposure (8, 9). In contrast, our findings are more in line with a report that claims 36% of methylation sites remain methylated in the absence of MET1 activity; because MET1 is less expressed, hypomethylation of DNA is more plausible (34). Although not completely inactive in the roots of ten-week-old pines, a lowered expression of MET1 would still entail a decrease in the overall methylation of methylated sites. A possible explanation for the absence of the upregulation of MET1 can be that the dose rate that was applied (682 µGy/h) is too low to incur such effects as observed in the field. Interestingly, the downregulation of MET1 implies an increase in transposon mobilisation. Due to the lowered grade of methylated regions, transposons are allowed to jump more freely (23). The combined dysfunctionality of MET1 and CMT3 (maintenance methylation enzyme of CHG sites) has been shown to result in a strong DNA hypomethylation in both CG and CHG contexts, accompanied by an elevation of transposition (23, 81). However, we never observed any changes in the level of expression of CMT3 despite also being responsible for methylation maintenance like MET1. We also were unable to confirm previous results that showed an upregulation of MET1 in the old needles of 10-week-old Scots pines (pers. comm. Brix De Rouck) (82). This may be due to the use of different climate chambers with different light sources for the cultivation of pines, which may lead the pines from both cultivations to be at different growth stages.

Hypermethylation is typically associated with an upregulation of *de novo* methylation enzymes like DRM2 (9). Interestingly, no higher expression of DRM2 was found either. In line with this idea, ROS1 should be downregulated as well since it is responsible for demethylation in plants (37). But also in this case, no difference in expression was encountered. Even more so, DNA methylation has been found to be more crucial for environmental stress responses in plants that have complex

genomes (23). Given the large size of the genome of pines, we expected an increase in expression for all methylation enzymes (59). This begs the question of why MET1 was significantly downregulated while other methylation enzymes were not.
A first plausibility is that we have included too few replicates in our analysis, resulting in a too-low power of statistical tests to reveal a population-representative effect for all methylation enzymes. This would suggest we were not able to picture the actual effects of radiation on the expression of methylation enzymes. Including more replicates in the RT-qPCR analysis may help generate more accurate results representative of the entire population of cultivated Scots pines. Secondly, it may be possible the plant experiences too much stress from additional stressors like ROS as mentioned before. As a result, the pines may invest more resources in the altered expression of genes involved in its defensive mechanisms. Yet, because methylation genes are closely involved in protecting DNA from radiation damage by altering its structure (23), and because hypermethylation was observed in the irradiated field population of pines (8), upregulation of these genes was expected. Lastly, irradiated pines may lower the expression of MET1 in their roots because they simply do not need to maintain their methylation due to the low mitotic activity of cells in the roots. Normally, MET1 is involved in converting hemimethylated copies of the genome after cell division back into symmetrically methylated DNA before the next S-phase (83). A decrease in cell division may also be a protective measure against radiation damage, resulting in the observed lighter roots at week 10 (Figure 4.B and Table 2).

It remains unclear why MET1 is less expressed in the roots of 10-week-old pines, especially considering this is the tissue most sensitive to radiation according to the lower mean root weight (Figure 4.B and Table 2). To investigate this question further, more research is needed. Perhaps including other GOIs involved in DNA methylation or more replicates may prove beneficial. Measuring the global DNA methylation levels or specific differentially methylated regions might also be of interest. Again, a different cultivation setup that eliminates the possibility of ROS being produced in the medium of pines may bring more clarity to the data presented here. To summarise, pines that have been irradiated for ten weeks seem to lower the expression of MET1, for a reason that remains unknown.

Expression tendencies of sirtuins
Other trends of borderline insignificant differences in expression are only to be found in the old needles at week 6 and week 10 (Suppl. E and F). The slight upregulation of SRT1 in the old needles at week 6, could indicate a response to stress because evidence suggests SRT1 has an important role in safeguarding against genome instability, cell damage and oxidative stress in rice (23). Given the possibility ROS may be produced by gamma radiation-induced hydrolysis in the hydroculture medium, an increase in SRT1 expression may be beneficial if the same protective properties of SRT1 are apparent in pines as they are in rice. However, SRT1 is only higher expressed in the old needles, instead of the root tissue which would be directly exposed to the ROS. The upregulation of SRT1 is also not statistically significant ($p = 0.057$), thus it does not indicate a strong effect. Looking at the scale of the relative expression plot (Suppl. F), the trend of upregulation of SRT1 might not mean anything: the biological relevance of changes smaller than a $\log_2$ 1-fold can be debated.

The tendency of SRT2 to be slightly less expressed in the old needles and roots at week 10 follows closely in the differences in expression as observed for MET1. Both SRT2 and MET1 are less expressed in these tissues, yet they have very different functions. SRT2 is responsible for the deacylation of histones, directly altering the 3D-structure and availability of chromatin for gene expression and other DNA-related processes (40, 41). The slightly lower expression of SRT2 would entail a lower need for SRT2 to deacylate histones, rendering more of the loose chromatin vulnerable to ionising radiation, but also more available for DNA transcription. There are two ways of looking at this observation: first, it may be possible that the exposure to a dose rate of 682 µGy/h is not high enough to induce actual DNA damage. Most of the chromatin remains loose and not densely packed as euchromatin in the nucleus. DNA transcription in irradiated plants may occur unaltered compared to control plants. On the other hand, the slight downregulation of SRT2 may result in more

vulnerable DNA because there is an active thread of ionising radiation-induced DNA damage, but also an increase in the availability of DNA for transcription (and expression) of genes involved in defensive mechanisms against radiation damage. We hypothesised the expression of methylation enzymes and sirtuins may be increased due to exposure to ionising radiation precisely because of their involvement as defensive actors in the protection of DNA against radiation (29). Hence, we believe the first explanation to be more plausible: the dose rate of 682 µGy/h is not high enough to incur changes in the gene expression of methylation enzymes and sirtuins. Although changes in gene expression have been proven in literature at lower dose rates in the field (22), we cannot confirm such observations in a laboratory cultivation. Perhaps the discrepancy between field and laboratory studies is greater than may be lead on. In any case, we must conclude no significant alteration in the expression of sirtuins was observed at any time during a 10-week laboratory cultivation of Scots pines.

CONCLUSION

Taking together all data presented in this thesis, it can be stated that chronic exposure to ionising radiation with a dose rate of 682 µGy/h has a significant effect on the growth of Scots pines, mainly in terms of root length and weight. However, the meristem of roots does not seem negatively affected by ionising radiation. Instead, a growth deviation is visible in the shoot meristem which is more globularly shaped and lacks leaf primordia.

In terms of gene expression, MET1 was downregulated in the roots of ten-week-old irradiated Scots pine seedlings. While we expected upregulation of methylation enzymes, the opposite was observed, but only for MET1. This downregulation implies there is a change in genome-wide DNA methylation in the roots of ten-week-old Scots pines, although further research is needed to investigate this assumption.

Lastly, an attempt was made to compose a nuclei extraction and staining protocol. The choice of extraction buffer requires more investigation to determine the best-suited buffer for extracting nuclei from pine tree tissue. It is encouraged to follow up on the data presented here with more elaborate and different experimental setups to eliminate any obscurity from the presented results in this thesis. We believe these results may serve to guide researchers in their experimental procedures for non-model organisms like Scots pine, as many techniques discussed here are relevant and novel in the context of biosphere impact studies.

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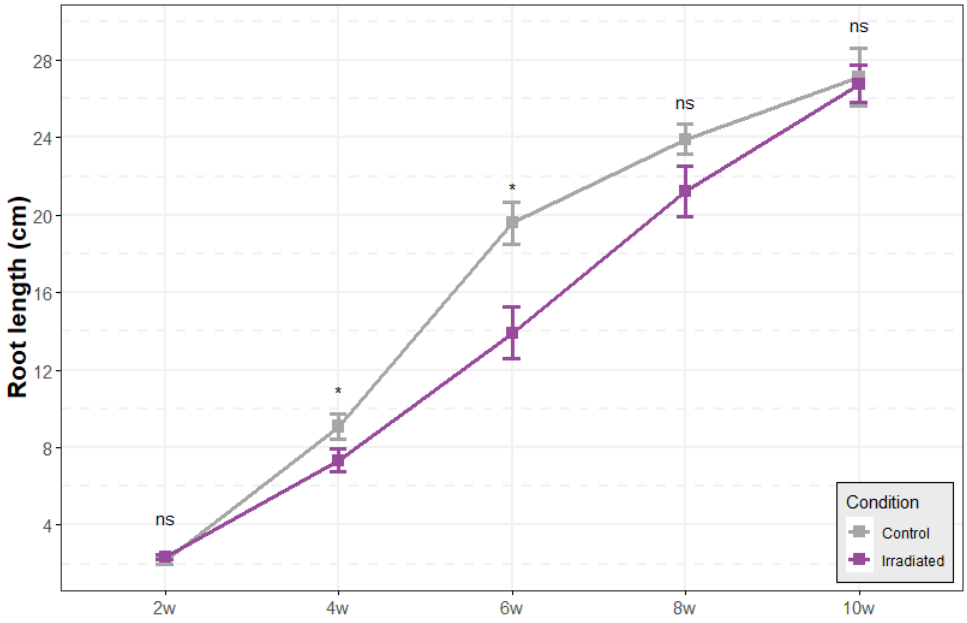
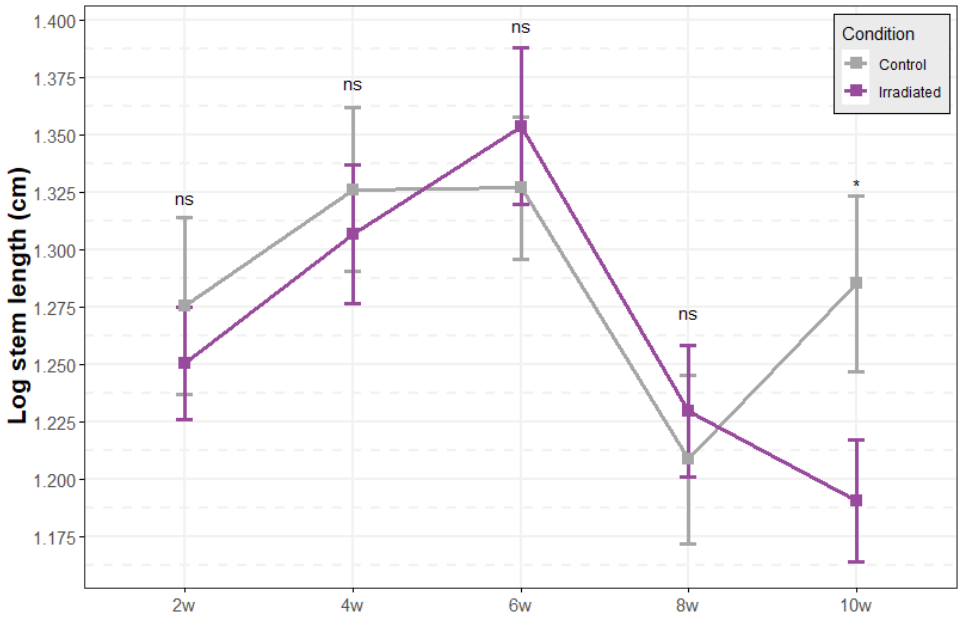
SUPPLEMENTALS

Supplement A Primer selection criteria on which the primers in Table 1 have been selected. For reference, see https://www.ncbi.nlm.nih.gov/tools/primer-blast/.

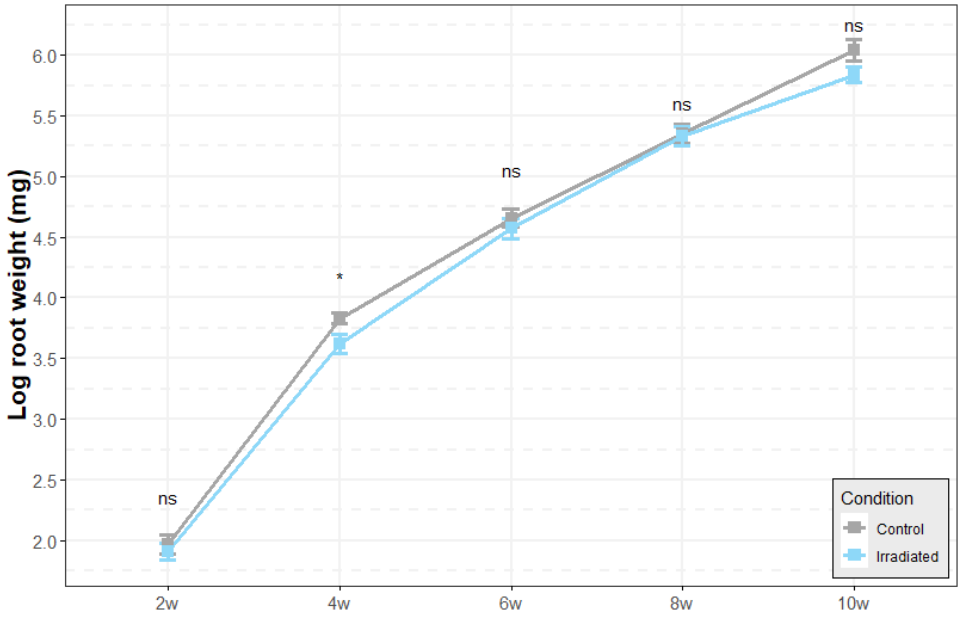
Criterion	**Value**		
PCR product size	**min** 80 bp		**max** 150 bp
Primer melting temperatures (T_m)	**min** 57 °C	**optimal** 60°C	**max** 63 °C
Max T_m difference	3°C		
Primer size	**min** 18 bp		**max** 24 bp
Primer GC% content	**min** 40%		**max** 60%
Max Poly-X (X = G or C)	4		
Max GC in primer 3'	4		
Max self-complementarity	**any** 4		**3'** 4
Max pair-complementarity	**any** 4		**3'** 4



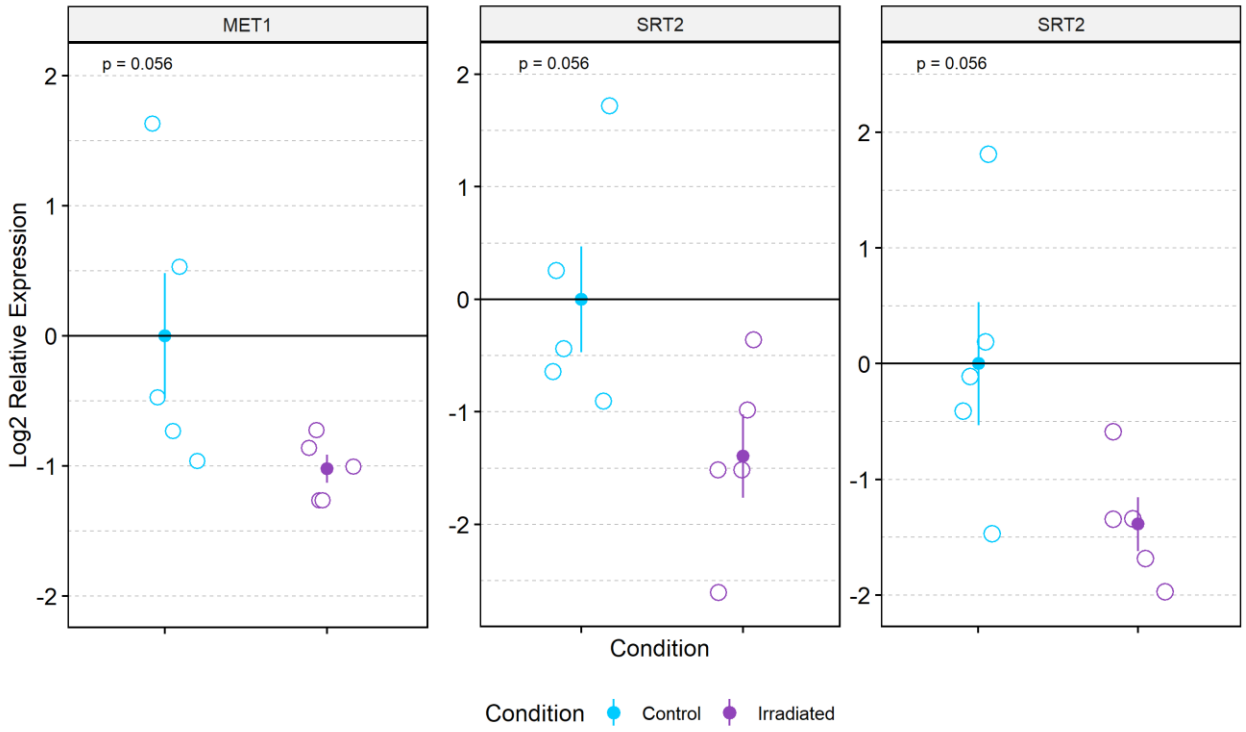
Supplement B Lineplot of the root length (in cm). Significance levels were obtained by using a Wilcoxon rank-sum test to compare the control and irradiated groups per timepoint.



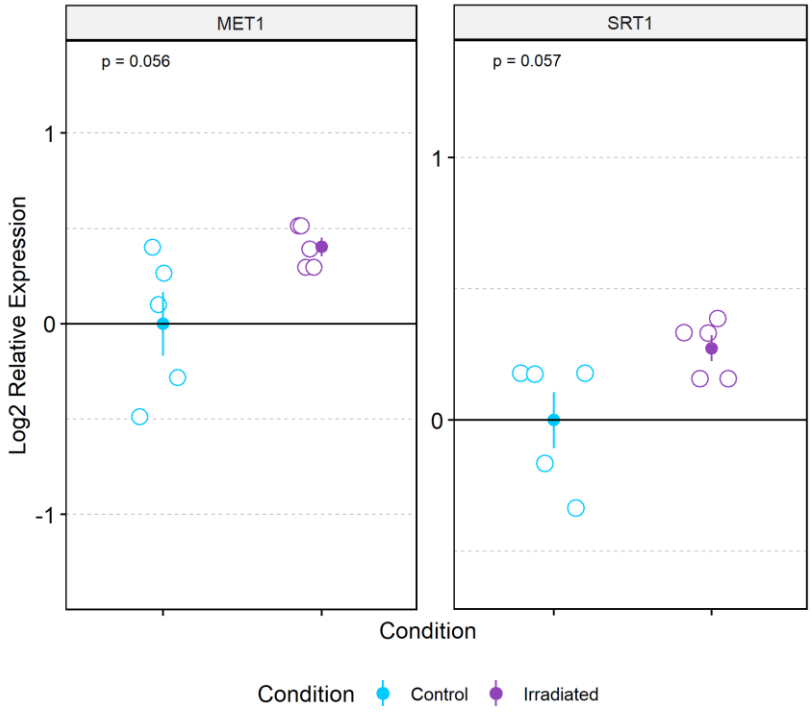
Supplement C Lineplot of the log stem length (in cm). Significance levels were obtained by using a t-test to compare the control and irradiated groups per timepoint.



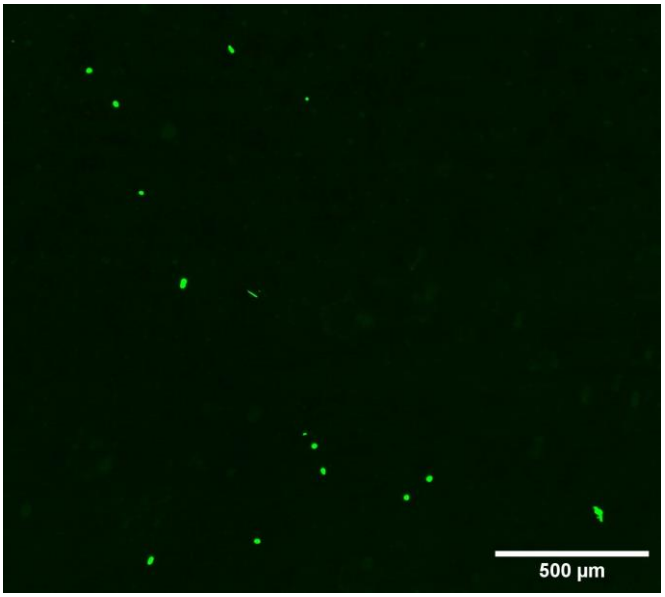
Supplement D Lineplot of the log root weight (in mg). Significance levels were obtained by using a t-test to compare the control and irradiated groups per timepoint.



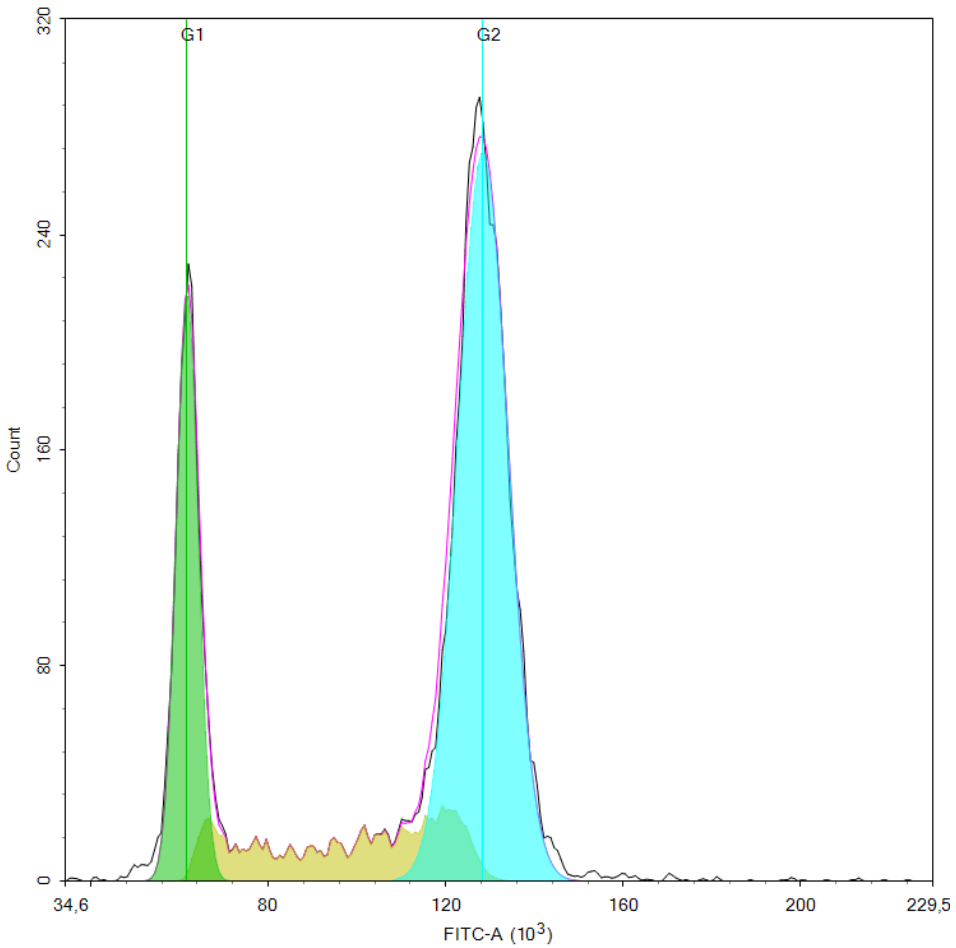
Supplement E Relative expression of MET1 in old needles, SRT2 in old needles and SRT2 in roots at week 10 respectively from left to right.



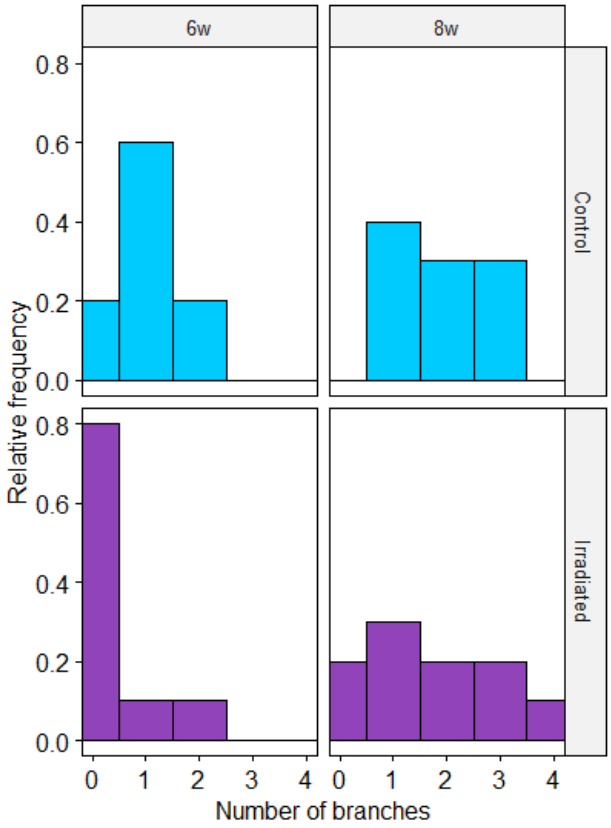
Supplement F Relative expression of MET1 and SRT1 in old needles at week 6.



Supplement G Fluorescence microscopy visualisation of SYBR Green stained pine tree nuclei extracted with WPB from 80 mg of 7 week old shoot tissue.



Supplement H Cell cycle plot generated using the NovoCyte Quanteon software based on the cloud of events visualised around a FITC-signal of 10^{5th} and FSC-signal between 10^4 and 10^5 (Figure 1).



Supplement I Relative frequency of the number of branches per plant for the 6 and 8 week timepoint. A χ^2-test was performed comparing the control and irradiated groups per timepoint (week 6 p = 0.01725; week 8 p = 0.5989).