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Research paper

Chronic exposure to ionizing radiation elicits growth inhibition and a dynamic oxidative stress response in the shoots of scots pine (*Pinus sylvestris*) seedlings

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ABSTRACT

Radioactive contamination represents a significant environmental stressor and can result in long-term exposure of terrestrial ecosystems to harmful levels of ionizing radiation. While Scots pine (*Pinus sylvestris*) has become a cornerstone reference species for environmental radiation protection studies due to its pronounced radiosensitivity, the underlying physiological mechanisms governing its responses to chronic low-dose radiation exposure remain poorly characterized. We therefore investigated the effect of chronic gamma irradiation on the growth and the oxidative stress response of young pine seedlings. The plants were exposed to 682 $\mu Gy \cdot h^{-1}$ for 10 weeks, with phenotypical measurements and analysis of molecular and biochemical markers of oxidative stress measured at two-week intervals. Chronic exposure induced significant inhibition of shoot development and a transient reduction of axillar bud formation, correlating with a dynamic oxidative stress response. An initial stress phase involved a significant disruption of the antioxidant system, evidenced by elevated glutathione oxidation and suppressed antioxidative enzyme activities. Despite this disruption, seedlings demonstrated the capacity for swift stress acclimation, first re-establishing glutathione homeostasis through an increase in its reduced form and then transitioning to an enhanced defensive state characterized by elevated superoxide dismutase activity and increased expression of ascorbate peroxidase. Understanding the dynamic radiation stress response during seedling establishment of this keystone forest species may prove beneficial for predicting ecosystem resilience and informing environmental radiation protection strategies in radioactively contaminated environments.

1. Introduction

Ionizing radiation (IR) refers to electromagnetic waves or subatomic particles with sufficient energy to remove electrons from atoms, thereby ionizing them. As a result, IR can damage key biomolecules such as DNA, proteins and lipids, making it harmful to living organisms. Naturally occurring IR, originating from cosmic rays or radioactive isotopes in the Earth's crust, constitutes a primordial environmental stressor that may have influenced the early evolution of life (Duarte et al., 2023). However, human technological advancements over the past century have introduced substantial levels of radioactivity into the environment, posing a significant threat to ecological stability (Geras'kin, 2016). Major environmental contamination with radiological sources occurred as accidental releases from nuclear incidents, such as Mayak (1957), Chornobyl (1986), and Fukushima (2011). The Chornobyl and Fukushima accidents are the only two events classified at the highest nuclear event severity level, INES 7 (International Nuclear and Radiological Event Scale, 2019), with Chornobyl widely regarded as the most severe nuclear disaster to date. The Chornobyl incident released approximately 5300 PBq of radioactive material, resulting in both acute radiation exposure of local biota during the early phase, and persistent environmental contamination with long-lived radionuclides such as ^{137}Cs and ^{90}Sr (Steinhauser et al., 2014).

Scots pine (*Pinus sylvestris* L.) dominated the forest surrounding the

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Chornobyl Nuclear Power Plant. Pine trees have long been recognized as one of the most radiosensitive plant taxa, potentially due to the large amount of genetic material in their cells (Amiro and Sheppard, 1994; Sparrow and Miksche, 1961; Sparrow and Woodwell, 1962). They are considered a reference organism by the International Commission on Radiological Protection (ICRP, 2008) and have demonstrated potential as a bioindicator of environmental radioactive contamination (Geras'kin et al., 2019). Their high radiosensitivity relative to other plant species has been confirmed in recent laboratory studies (Bhattacharjee et al., 2023; Blagojevic et al., 2019a) and was dramatically illustrated during the acute radiation exposure following the Chornobyl accident. This event caused mass mortality, tissue damage, growth inhibition, and reduced fecundity in pines near the reactor (the so-called "Red Forest" area), while nearby deciduous trees were less affected (Arkhipov et al., 1994).

Long-term radiation exposure in the decades following the Chornobyl and Fukushima accidents has also led to significant biological effects in conifer species. Notably, Chornobyl pines have exhibited radiation-induced loss of apical dominance, a phenomenon also observed in pines and other conifers growing in the Fukushima-affected zone (Nybakken et al., 2023; Watanabe et al., 2015; Yoschenko et al., 2016, 2011). Similarly, increased needle necrosis and altered needle morphogenesis have been reported in both regions (Makarenko et al., 2021, 2016). Microscopic analyses of apical needles from Chornobyl pines revealed elevated numbers of resin ducts in high-radiation areas, along with various organelle abnormalities (Nybakken et al., 2023). Cytogenetic abnormalities in the meristems of chronically irradiated pines have also been frequently observed (Geras'kin et al., 2021; Vasiliev et al., 2020; Yoschenko et al., 2011). At the molecular level, effects include DNA damage (Nybakken et al., 2023) and increased genetic diversity (Geras' kin et al., 2010; Geras'kin and Volkova, 2014; Kuchma and Finkeldey, 2011; Volkova et al., 2017), as well as transcriptomic (Bitarishvili et al., 2024; Duarte et al., 2019), metabolomic (Bitarishvili et al., 2024, 2021; Nybakken et al., 2023), and epigenomic alterations (Bondarenko et al., 2023; Kovalchuk et al., 2003; Volkova et al., 2018). Chronic irradiation has also been shown to induce transgenerational effects in pines: seeds derived from contaminated plots yielded seedlings with greater shoot length and altered antioxidant capacity, and an additional irradiation treatment resulted in enhanced root growth and reduced DNA damage compared to naive controls (Rahman, 2021).

To gain mechanistic insights, controlled laboratory studies have investigated radiation responses in conifers. Multi-day (chronic) gamma irradiation of Scots pine seedlings has been shown to cause dose-dependent growth inhibition, alterations in shoot apical meristem morphology, and transcriptional changes (Blagojevic et al., 2019a). Chronic IR exposure has also been shown to induce dose-dependent DNA damage that may persist even several weeks after the irradiation period (Blagojevic et al., 2019a; Rahman, 2021), and elevated hydrogen peroxide levels have also been observed following such treatments (Blagojevic et al., 2019b). Norway spruce (*Picea abies*), another member of the Pinaceae, is another conifer studied under laboratory irradiation. Its radiosensitivity has been shown to be comparable to that of pine (Blagojevic et al., 2019a). At low dose rates, *P. abies* seedlings exhibited DNA damage and limited transcriptional changes without growth inhibition, whereas higher dose rates significantly reduced growth and triggered widespread gene expression changes indicative of a stress response (Blagojevic, 2019). Interestingly, the model plant *Arabidopsis thaliana*, which is considered radioresistant, exhibited a greater transcriptomic response than *P. abies* as far more genes were differentially expressed upon irradiation and this response was induced at lower dose rates than in *P. abies* (Bhattacharjee et al., 2023). These findings led the authors to propose that the high radiosensitivity of conifers such as pines may result from a limited stress response.

While laboratory exposure studies are crucial for increasing our mechanistic understanding of how radiation fundamentally affects plant growth, they often focus on acute and/or relatively high radiation exposures (Mishra et al., 2024). Hence, fundamental knowledge on how plants respond to chronic low-level radiation exposures as found in nuclear accident affected areas is still largely lacking (Caplin and Willey, 2018). The plant response to acute versus chronic radiation exposure is often fundamentally different (Choi et al., 2021; Goh et al., 2014; Hong et al., 2022, 2018; Kovalchuk et al., 2007). However, cellular damage is induced in a similar manner in both cases. Direct damage may occur due to ionization of biomolecules such as DNA, while IR-induced production of harmful reactive oxygen species in the cells can lead to indirect damage (Duarte et al., 2023). Specifically, radiation can induce water radiolysis, generating reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), superoxide (O_2^{-}•), and hydroxyl (OH•) radicals (Esnault et al., 2010). This ROS accumulation can damage biomolecules and lead to oxidative stress (Einor et al., 2016). Yet, ROS also function as signaling molecules that mediate stress responses, and plants possess an intricate metabolic network to regulate ROS levels, involving both enzymatic and non-enzymatic scavenging systems (Wang et al., 2024).

Key antioxidant enzymes include superoxide dismutase (SOD) and catalase (CAT), which reduce ROS independently of secondary metabolites through SOD-mediated conversion of superoxide radicals into H_2O_2 and its further decomposition into H_2O and O_2 by catalase (Ahmad, 2014). Plant SODs exist in multiple families: copper/zinc-binding SOD (CSD) is the primary cytosolic form, while iron- and manganese-binding SODs operate mainly in chloroplasts and mitochondria, respectively (Alscher, 2002). The ascorbate–glutathione cycle is also central to ROS detoxification, and involves ascorbate peroxidase (APX) using ascorbate to reduce H_2O_2, producing dehydroascorbate (Foyer and Noctor, 2011). This is regenerated by dehydroascorbate reductase (DHAR) using reduced glutathione (GSH), which is oxidized to glutathione disulfide (GSSG). GSSG is recycled to GSH by glutathione reductase (GR) using NADPH (Ding et al., 2020). The GSH/GSSG balance is regulated through biosynthesis, catabolism, and redox cycling. Biosynthesis of GSH involves the rate-limiting γ-glutamylcysteine synthetase (GSH1) and GSH synthetase (GSH2; Dorion et al., 2021). Beyond its role in the ascorbate cycle, GSH can also directly detoxify H_2O_2 via glutathione peroxidases (Madhu et al., 2023). As a central antioxidant metabolite, glutathione plays a pivotal role in managing oxidative stress and is affected by a wide range of stressors (Dorion et al., 2021).

Both acute and chronic exposure to ionizing radiation are known to affect the oxidative stress metabolism in plants (Caplin and Willey, 2018; Esnault et al., 2010). Oxidative stress responses to chronic irradiation have been observed at the transcriptomic, enzymatic, and metabolite levels in various plant species, including *Arabidopsis thaliana* (Goh et al., 2014; Van De Walle et al., 2016; Vandenhove et al., 2010; Vanhoudt et al., 2014), *Lemna minor* (Van Hoeck et al., 2015a, 2015b; Xie et al., 2019), *Oryza sativa* (Choi et al., 2021; Hayashi et al., 2014; Kariuki et al., 2019; Rakwal et al., 2009), *Stipa capillata* (Zaka, 2002), *Viccia cracca* (Voronezhskaya et al., 2023), *Solanum lycopersicum* (Kim et al., 2021) and various herbaceous species growing in the Chornobyl Exclusion Zone (Volkova et al., 2021). However, these responses are highly dependent on the radiation dose rate and exposure duration, as well as the plant species, tissue type, and developmental stage. For example, oxidative DNA damage was induced by chronic irradiation in 10-day old *A. thaliana* seedlings but reduced when 14-day old seedlings were exposed (Biermans et al., 2015a). In *Pinus sylvestris*, chronic laboratory irradiation did not alter the expression of genes encoding CAT or SOD in the shoot and caused only minimal changes in peroxidase expression, while total antioxidative capacity also remained unaffected (Rahman, 2021). In contrast, field studies on Chornobyl pines indicate a distinct oxidative stress response. Transcriptomic analysis of trees growing in contaminated areas revealed modulation of glutathione biosynthesis and endogenous ROS production (Duarte et al., 2019), while biochemical measurements showed elevated GSH levels, reduced GSSG abundance, increased CAT activity, and decreased peroxidase activity (Volkova et al., 2017). These findings suggest that modulation of oxidative stress metabolism may represent an adaptive strategy to cope with chronic radiation in pines under field conditions.

While field studies suggest pine trees may alter their oxidative stress metabolism to adapt to chronic radiation exposure in the long term, the initial, dynamic processes that lead to this state remain unclear. We hypothesize that the pronounced radiosensitivity of *Pinus sylvestris* is not only due to a passive failure to cope with radiogenic ROS, but also reflects an active stress response that is temporarily overwhelmed. Specifically, we propose that chronic low-dose irradiation initially disrupts the antioxidant system, leading to an acute state of oxidative stress which likely contributes to growth inhibition. We further posit that seedlings actively overcome this initial shock through a compensatory increase in the reduced glutathione (GSH) pool, serving to re-establish redox homeostasis and allowing the plant to transition into an acclimated state characterized by enhanced, long-term anti-oxidative defenses. To test this hypothesis, we investigated the temporal dynamics of shoot growth and glutathione homeostasis as well as the activity and transcription of key antioxidant enzymes in pine seedlings throughout a 10-week chronic gamma irradiation treatment. This study thereby presents the first temporal analysis of glutathione metabolism as well as antioxidant enzyme activity and transcription in a conifer under chronic laboratory irradiation, addressing a critical knowledge gap in the radiation response of this key radiological reference organism.

2. Materials and methods

2.1. Pine seedling culture

Pinus sylvestris L. seeds (Pelgrum-Vink Material B.V., Lobith, The Netherlands) were imbibed overnight in distilled water and surface-sterilized by rinsing with 0.5 % NaOCl for one minute, followed by five rinses with autoclaved distilled water. Seeds were then placed in sterile Petri dishes containing moist filter paper (Grade 292, Sartorius, Cat. FT-3–205–125) and stratified in the dark for 7 days at 4 °C. After stratification, seeds were sown onto coarse vermiculite in 15 mL Falcon™ tubes, whose bottom halves were replaced with a metal mesh. The vermiculite was kept moist by suspending the modified tubes in distilled water. Four seeds were sown per tube, and 27 tubes were arranged in 1.5 L trays. After two weeks of germination, excess seedlings were removed to obtain a uniform set of one seedling per tube. At that point, distilled water was replaced with half-strength Hoagland solution (Hoagland and Arnon, 1938). From then onward, air was continuously supplied to the nutrient solution via silicone tubing connected to a peristaltic pump (1 rpm; Minipuls® Evolution, Gilson, USA). The solution was refreshed weekly throughout the experiment. Germination and subsequent plant growth were conducted in a climate chamber maintained at 24 °C/20 °C (day/night) and 30 % relative humidity. Seedlings were illuminated with Valoya LED Grow Lights (380–780 nm spectrum) under a 16/8 h photoperiod and an average PAR of 140 $\mu mol \cdot m^{-2} \cdot s^{-1}$ measured at plant level.

2.2. Irradiation treatment

Four trays, each containing 27 tubes, were subjected to chronic gamma irradiation beginning at the time of sowing. Irradiation was applied by placing a radiation source on two sides of each tray. Each source consisted of a laminated plastic plate coated on one side with a ^{137}Cs solution, yielding a total activity of 820 kBq per plate. It was assumed that beta particles emitted by the cesium did not significantly penetrate the laminating foil, and that seedlings were therefore primarily exposed to gamma radiation. The gamma dose rate at the shoot level was measured at 682 ± 74 $\mu Gy \cdot h^{-1}$ using eight thermoluminescent dosimeters placed around the culture setup. The control group consisted of four additional trays of 27 seedlings, separated from the treatment group by a 32 cm high, 6 cm thick lead wall. For the control group, non-radioactive laminated plates of identical appearance were placed on either side of each tray. The gamma dose rate at the shoot level was also measured in the control group using thermoluminescent dosimeters and did not differ significantly from the normal background radiation. Both treatment and control trays were enclosed within paraffin block walls (80 cm high, 16 cm thick) to shield personnel working in the climate chamber. Within each group, trays were regularly rotated and shuffled to homogenize light exposure; in the treatment group, this also served to equalize the average dose rate received by each seedling.

2.3. Phenotyping and tissue sampling

After 2, 4, 6, 8, and 10 weeks of growth, five randomly selected seedlings per tray (20 per condition) were harvested for phenotypic and molecular analysis. At the final timepoint, both the number of axillary buds and axillary branches per seedling were recorded, while at earlier timepoints only axillary buds were counted due to the absence of branch development. Seedlings were placed on a laboratory bench with roots outstretched and photographed for later measurement of root and shoot length. Fresh weights of shoot and root tissues were then recorded, and a sample of the young shoot tissue was immediately flash-frozen in liquid nitrogen and stored in microcentrifuge tubes (Eppendorf™ 1.5 mL Safe-Lock, Cat. #0030120086),. For the first two timepoints, entire shoots (6–10 young needles and the shoot meristem) were harvested, yielding approximately 35 mg of shoot tissue. For the final three timepoints, approximately 100 mg of shoot tissues (10–12 young needles and the shoot meristem) were collected. Frozen samples were stored at –80 °C until analysis. Tissue homogenization was performed using 3 mm glass beads (five or six per tube) in a Retsch® MM 400 cryomill operated at 30 Hz for 2 min, with pre-chilled tube holders. Total root length (from stem base to furthest root tip) and shoot length (from stem base to shoot apex) were quantified from seedling images using ImageJ (v1.54d). Enzyme activity analysis, glutathione measurements and gene expression analysis were each conducted using five biological replicates per timepoint/condition combination, obtained by randomly allocating five out of 20 samples harvested per timepoint/condition to each of the analyses.

2.4. Enzyme activity analysis

Total protein was extracted by adding 500 μL of extraction buffer (0.2 M Tris, 2 mM dithiothreitol, 1 mM EDTA, 2.5 % polyvinylpyrrolidone [average molecular weight 40,000], pH 8.4) to frozen, ground plant tissue, followed by vortexing until thawed. The 2.5 % polyvinylpyrrolidone was included to bind polyphenolic compounds present in the extract, which otherwise interfered with enzyme assays. The crude extract was centrifuged at 12,000 × g for 10 min at 4 °C, and the resulting supernatant was transferred to a fresh tube, kept on ice, and used undiluted in all enzyme assays. Enzyme activities of guaiacol peroxidase (GPOD), catalase (CAT), glutathione reductase (GR) and superoxide dismutase (SOD) activity were measured according to established methods (Bergmeyer, 1965; McCord and Fridovich, 1969). Protein concentration was quantified using Bradford (Bradford, 1976) Reagent (Sigma, Cat. B6916), and enzyme activities were expressed as enzyme units (U) per mg protein. All spectrophotometric assays were performed in triplicate using a PowerWave HT plate reader (BioTek) and Gen 5.11 software, in either polystyrene 96-well plates (Greiner Bio-One, Cat. 655 101) or UV-Star® 96-well plates (Greiner Bio-One, Cat. 655 801), depending on the absorbance wavelength.

2.5. Glutathione measurements

Reduced (GSH) and oxidized (GSSG) glutathione concentrations were determined spectrophotometrically following a previously described method (Horemans et al., 2015) with the following modifications: 1) An extraction volume of 200 μL 0.2 M HCl was used for shoot samples collected at the 2- and 4-week timepoints due to limited sample availability. The remainder of the protocol was scaled accordingly. 2)

For GSH precipitation, 1.3 μL of 2-vinylpyridine was added per 100 μL aliquot of the neutralized acid extract. All glutathione measurements were performed in triplicate.

2.6. Gene expression analysis

2.6.1. RNA extraction

Total RNA was extracted from frozen, ground plant tissue using the Qiagen RNeasy Plant Mini Kit, following the manufacturer's instructions with the following modifications: 1) Approximately 25 mg of insoluble polyvinylpyrrolidone (average molecular weight 40,000,) was added to the frozen sample before lysis buffer addition. 2) A lysis buffer volume of 900 μL was used instead of 450 μL. The lysate was passed through the spin columns in two sequential centrifugation steps to accommodate the increased volume. 3) RNA was eluted in two centrifugation steps using 15 μL RNase-free water for each step. RNA concentration and purity were assessed using a NanoDrop 2000 spectrophotometer, and samples were stored at −80 °C until further processing.

2.6.2. DNase treatment and cDNA synthesis

Prior to reverse transcription, RNA was treated with DNase using the TURBO DNA-free Kit (Ambion) according to the manufacturer's instructions and the following reaction mixture: 1 μL 10X reaction buffer, 0.25 μL DNase, and 1 μg RNA per reaction, diluted with RNase-free water to a final volume of 10 μL. Following DNase inactivation, 6.5 μL of treated RNA was recovered and used for reverse transcription. An additional 1 μL from each reaction was pooled to serve as a no-RT control during the real-time quantitative PCR (RT-qPCR) to confirm the absence of DNA contamination. Reverse transcription was performed in a 20 μL reaction using the PrimeScript 1st Strand cDNA Synthesis Kit (Takara Bio), following the manufacturer's protocol. The resulting cDNA was diluted to 100 μL with RNase-free water and stored at –80 °C until analysis.

2.6.3. RT-qPCR

RT-qPCR was performed using a QuantStudio 5 Real-Time PCR System (Applied Biosystems™) in MicroAmp Optical 384-Well Reaction Plates (Cat. 4309849). Each 5 μL reaction contained 2.5 μL PowerTrack™ SYBR Green Master Mix (Cat. A45012), 1.2 μL diluted cDNA, and 1.3 μL primer mix. The primer mix consisted of equal concentrations of forward and reverse primers, diluted to 3 μM with nuclease-free water and 6 × Yellow Sample Buffer (PowerTrack™ kit). All reactions were run in duplicate. PCR cycling was conducted in fast mode under default conditions: 2 min at 95 °C for enzyme activation, followed by 40 cycles of 5 s at 95 °C and 30 s at 60 °C. A dissociation curve analysis followed the PCR, with temperatures ranging from 60 °C to 95 °C at a ramp rate of 0.075 °C/s. Ct values were calculated in QuantStudio Design & Analysis Software v1.5.2 using ROX as the passive reference dye and exported without additional processing. Relative expression levels were determined in RStudio (v2023.03.1) using R (v4.3.0). After averaging technical replicates, relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) with normalization to the geometric mean of all reference gene Ct values (Vandesompele et al., 2002), and expression was computed relative to the control at the first timepoint. Primer sequences, efficiencies, and target/reference gene details are provided in Table S1.

2.7. Statistical analysis

Statistical analyses were performed in RStudio (v2023.03.1) using R version 4.3.0. Axillary bud and branch counts between treated and untreated seedlings were compared at each timepoint using the Wilcoxon–Mann–Whitney test (Mann and Whitney, 1947). For all other data, linear models were fitted, and pairwise comparisons were performed using estimated marginal means with Tukey's adjustment for multiple testing, implemented via the emmeans package v1.10.6 (https://CRAN.R-project.org/package=emmeans).

3. Results

3.1. Irradiation-induced growth inhibition and altered root growth kinetics

The development of shoot and roots of pine seedlings exposed chronically to 682 $\mu Gy\cdot h^{-1}$ was followed from germination up to 10 weeks of development and compared to unexposed control conditions. Starting at week 6, irradiated seedlings tended to exhibit lower shoot fresh weight than controls, and this difference became statistically significant by week 10 (Fig. 1A). At week 10, control shoots weighed 604 ± 38 mg, whereas irradiated shoots averaged 510 ± 25 mg—a reduction of roughly 16 %. Similarly, root fresh weight declined from 450 ± 37 mg in the control to 363 ± 28 mg in the irradiated seedlings at week 10, representing a 19 % decrease (Fig. 1B). Shoot length (Fig. 1C) also showed a trend toward inhibition in the irradiated group beginning at week 6; however, none of these differences reached statistical significance ($p = 0.079–0.129$ at weeks 6–10). By contrast, root length (Fig. 1D) was significantly lower in the irradiated group at weeks 4 and 6. Control roots elongated rapidly from week 2 to week 6, then continued more gradually from week 6 to week 10. In contrast, irradiated roots elongated at a relatively constant, linear rate throughout the 10-week period. Consequently, although the irradiated roots eventually caught up to the controls by weeks 8 and 10, the roots of irradiated seedlings were significantly shorter at weeks 4 ($p < 0.05$) and 6 ($p < 0.0001$) compared to controls.

3.2. Irradiation affects axillar bud formation and branch development

Axillar bud formation started to occur after four weeks of seedling growth. At the first bud count after the onset of bud formation (six weeks), the irradiated seedlings had on average a significantly lower number of buds per seedling compared to the control. However, this effect was no longer present at later timepoints (Fig. 2A). The first development of buds into lateral branches started to occur after 8 weeks, and was therefore only measured at the end of the experiment. The resulting data indicate that irradiated seedlings had a significantly lower amount of branches after 10 weeks of irradiation, compared to the control (Fig. 2B).

3.3. Changes to shoot glutathione levels due to radiation treatment

Glutathione homeostasis in young shoots was markedly altered by irradiation (Fig. 3). By week 8, reduced glutathione (GSH) exhibited opposite trends: in control shoots, GSH concentration declined sharply, whereas in irradiated shoots it increased, resulting in a significant difference between treatments at that timepoint (Fig. 3A; $p < 0.01$). Oxidized glutathione (GSSG) remained stable over time in control seedlings but was significantly elevated in irradiated seedlings at weeks 6 and 8 relative to earlier timepoints and to the respective controls. By week 10, GSSG levels in irradiated shoots returned to control values (Fig. 3A). Glutathione oxidation (percentage of GSSG relative to total glutathione) is shown in Fig. 3B. In control seedlings, oxidation levels increased gradually from weeks 2–8 before decreasing again towards week 10. In irradiated seedlings, a similar pattern occurred but progressed more rapidly, as oxidation rose sharply between weeks 4 and 6. As a result, percent GSSG was significantly greater in treated versus control seedlings at week 6. By week 8, the gradual increase in the control resulted in comparable levels for both conditions, and by week 10 oxidation levels were essentially identical.

3.4. Chronic irradiation affects anti-oxidative enzyme activity

In young shoots, chronic exposure of young pine seedlings also

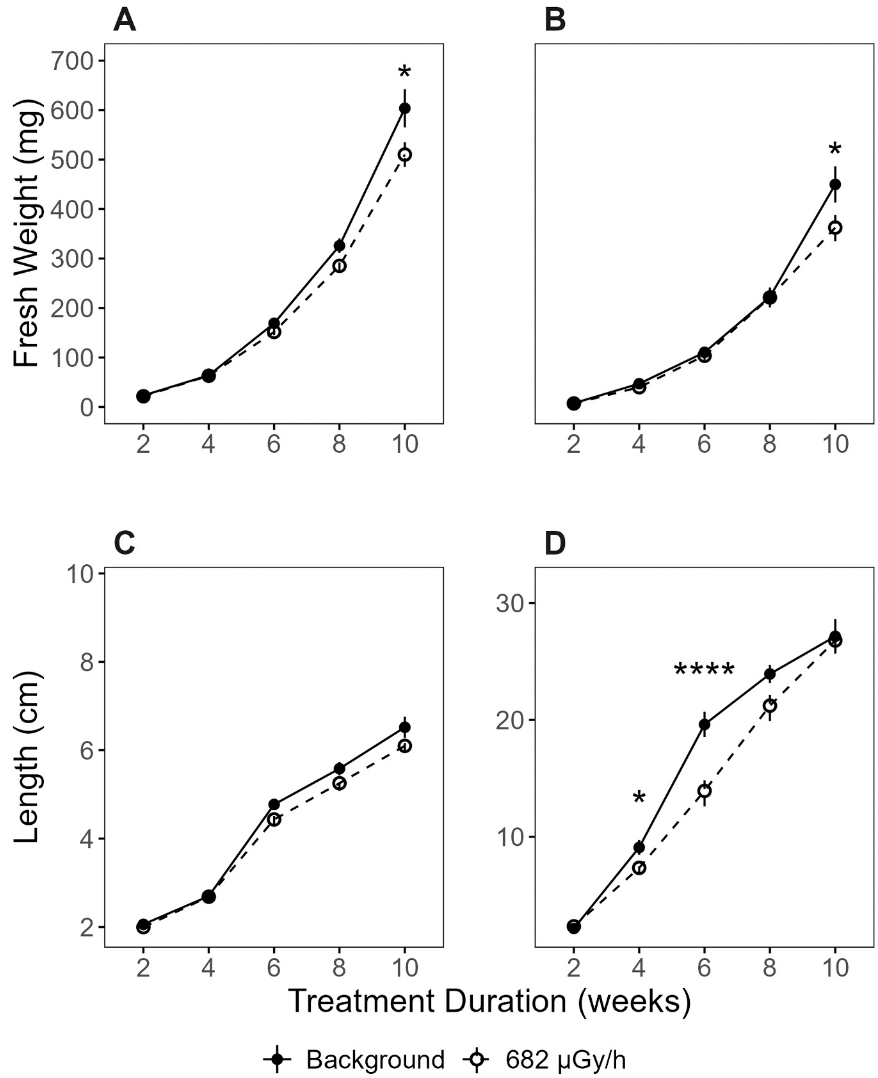

Fig. 1. Effects of chronic gamma irradiation on pine seedling growth. Phenotypical parameters of pine seedlings continuously exposed to external gamma irradiation at 682 μGy·h^{-1} (open symbols, dotted lines) compared to controls exposed to normal background radiation (solid symbols, solid lines) over the course of 10 weeks. Mean data (n = 20) is represented with the standard error. Significant differences between the two conditions are indicated as * ($p < 0.05$) and **** ($p < 0.0001$) using estimated marginal means and Tukey's correction for multiple comparisons. A) Shoot fresh weight (mg). B) Root fresh weight (mg). C) Shoot length (cm). D) Root length (cm).

induced significant, time-dependent effects on key oxidative stress enzymes (Fig. 4), namely glutathione reductase (GR), superoxide dismutase (SOD), and guaiacol peroxidase (GPOD). In both control and irradiated shoots, GPOD activity rose sharply between weeks 2 and 4 before declining toward week 6 and stabilizing thereafter. The decline at week 6 was more pronounced under irradiation, producing significantly lower GPOD activity than in controls at that timepoint ($p < 0.05$). GR activity increased from weeks 2–6 in both groups, but the magnitude of increase was smaller in irradiated seedlings. Consequently, GR activity at week 6 was significantly lower in the irradiated group ($p < 0.01$). Control seedlings displayed a sharp GR peak at week 6 followed by significant declines at weeks 8 and 10. In contrast, irradiated seedlings maintained a constant GR activity level from week 6 through week 10. In control shoots, SOD activity increased nearly linearly during the first eight weeks, then dipped slightly by week 10. Meanwhile, SOD activity in irradiated seedlings remained relatively low until week 6 and then rose rapidly, matching control levels by week 8 and surpassing them by week 10. As a result, SOD activity was significantly lower at week 6 in irradiated seedlings ($p < 0.01$) but higher after 10 weeks of treatment ($p < 0.01$). To summarize, all three enzymes examined exhibited significantly lower activity in irradiated seedlings than in controls at week 6, while by week 10, SOD activity in the irradiated group exceeded control levels.

3.5. Limited effect of irradiation on expression of key oxidative stress-related genes

We next examined whether chronic irradiation affected expression of key oxidative stress-related genes (Fig. 5), being *CATALASE 1* (*CAT1*), *CATALASE 3* (*CAT3*), *COPPER/ZINC SUPEROXIDE DISMUTASE 1* (*CSD1*), *COPPER/ZINC SUPEROXIDE DISMUTASE 3* (*CSD3*), *GLUTATHIONE REDUCTASE 1* (*GR1*), *GLUTATHIONE REDUCTASE 2* (*GR2*), *GLUTAMATE-CYSTEINE LIGASE* (*GSH1*), *GLUTATHIONE SYNTHETASE* (*GHS2*), *ASCORBATE PEROXIDASE* (*APX*) and *DEHYDROASCROBATE REDUCTASE 1* (*DHAR1*). In general, most transcripts were not significantly altered by irradiation at the tested timepoints. Neither *GR1* nor *GR2* showed consistent treatment effects: *GR1* remained stable in both groups, and *GR2* declined comparably over time in irradiated and control shoots. Similarly, expression of the biosynthetic enzymes *GSH1* and *GSH2* did not differ between treatments at any timepoint. *DHAR1*

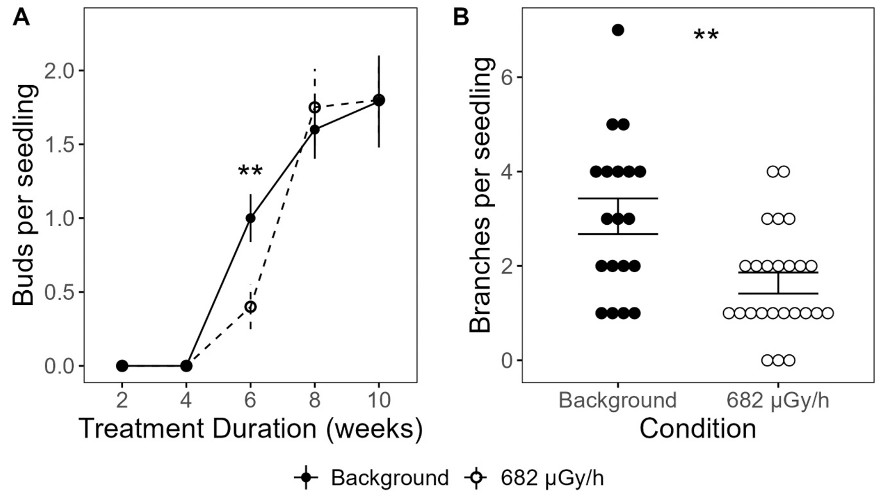

Fig. 2. Effects of chronic gamma irradiation on pine seedling shoot branching. Branching behavior of pine seedlings continuously exposed to external gamma irradiation at 682 µGy·h^{-1} (open symbols, dotted lines) compared to controls exposed to normal background radiation (solid symbols, solid lines). A) Number of axillar buds formed over the course of 10 weeks. Mean data (n = 20) is presented with the standard error. Significant differences between the two conditions are indicated as ** ($p < 0.01$, estimated marginal means and Tukey's correction for multiple comparisons). B) Number of axillar branches developed per seedling after 10 weeks, with each dot representing a single seedling. Error bars indicate mean ± standard error for the condition. A significant difference between the two conditions is indicated by ** ($p < 0.01$, Wilcoxon's rank-sum test).

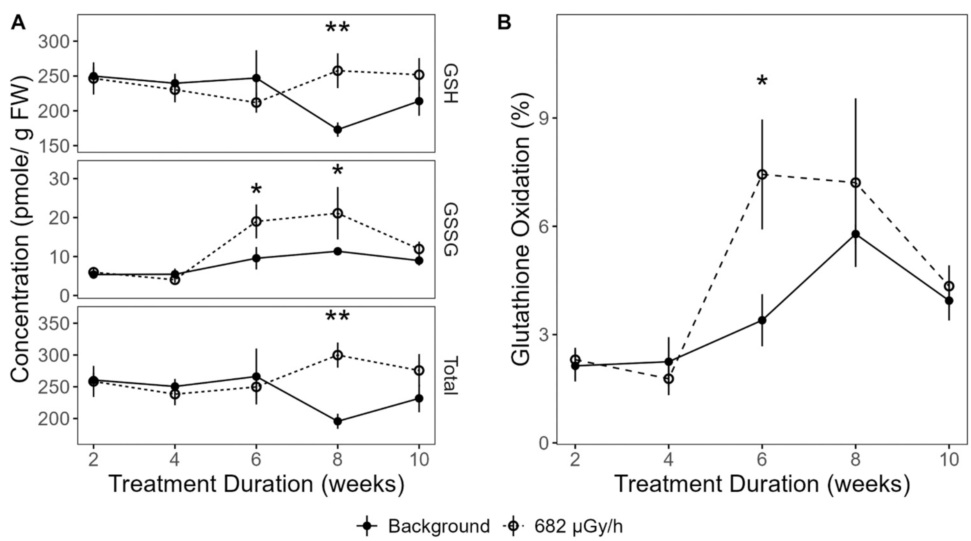

Fig. 3. Effects of chronic gamma irradiation on pine shoot glutathione levels. Glutathione levels in the shoots of pine seedlings continuously exposed to external gamma irradiation at 682 µGy·h^{-1} (open symbols, dotted lines) compared to controls exposed to normal background radiation (solid symbols, solid lines). Mean data (n = 5) is presented with error bars indicating standard error. Significant differences between the two conditions are indicated as * ($p < 0.05$) and ** ($p < 0.01$) using estimated marginal means and Tukey's correction for multiple comparisons. A) Concentration of reduced (GSG), oxidized (GSSG) and total (GSH + GSSG) glutathione. B) Oxidized glutathione expressed as a percentage of total glutathione.

expression rose over the experiment in both groups but was never significantly different between irradiated and control shoots. In contrast, *APX* exhibited a treatment-specific change: by week 10, irradiated seedlings maintained higher APX transcript levels than controls ($p < 0.05$), even though both groups trended downward before week 8. Both *CSD1* and *CSD3* displayed opposing temporal trends—*CSD1* gradually increased, *CSD3* declined—yet neither showed a significant irradiation effect. *CAT1* remained unaffected by treatment. *CAT3* showed a transient elevation in irradiated shoots at week 4 ($p < 0.05$), but no sustained difference thereafter. Overall, the only irradiation-dependent changes observed were a transient increase in *CAT3* expression mid-experiment and a late rise in *APX* expression under irradiation, while other oxidative stress–related genes were unaffected by chronic gamma exposure.

4. Discussion

The primary aim of this study was to assess the hypothesis that the oxidative stress response of pine seedlings to chronic irradiation is a dynamic, multi-phase process of disruption, compensation, and acclimation rather than simply stress accumulation. Our temporal analysis of phenotypic, biochemical and molecular endpoints provide some support for this mechanistic model. The following sections will discuss how the observed growth inhibition and the intricate dynamics of key antioxidant systems corroborate the distinct stages of the plant's response.

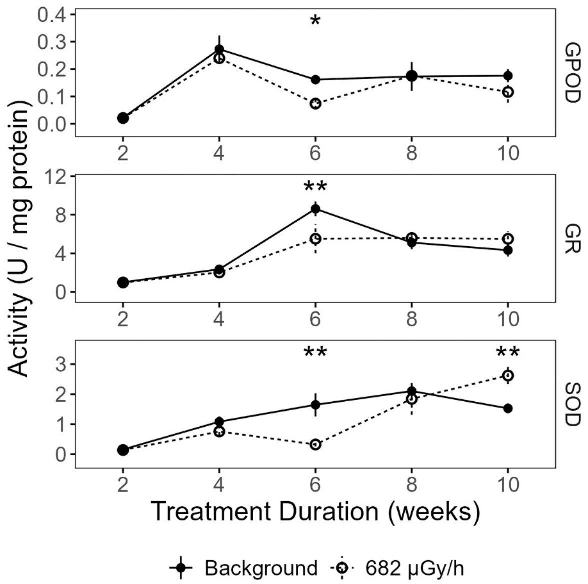

Fig. 4. Effects of chronic gamma irradiation on antioxidative enzyme activity in pine seedling shoots. Activity of antioxidative enzymes guaiacol peroxidase (GPOD), glutathione reductase (GR) and superoxide dismutase (SOD) in the shoots of pine seedlings continuously exposed to external gamma irradiation at 682 μGy·h^{-1} (open symbols, dotted lines) compared to controls exposed to normal background radiation (solid symbols, solid lines) over the course of 10 weeks. Mean data (n = 5) is presented with error bars indicating standard error. Significant differences between the two conditions are indicated as * ($p < 0.05$) and ** ($p < 0.01$) using estimated marginal means and Tukey's correction for multiple comparisons.

4.1. Chronic irradiation affects shoot growth and development in young pine seedlings

Chronic irradiation at a relative low dose rate for Scots pine (i.e. 682 μGy·h^{-1}) clearly affected shoot development in young pine seedlings, with shoot fresh weight and length declining from week 6 onward and a significant reduction in fresh weight observed after 10 weeks (Fig. 1A, C). This observation reinforces the well-established radiosensitivity of *Pinus sylvestris*, a trait first linked to its large nuclear volume decades ago (Sparrow and Miksche, 1961) and tragically illustrated by the acute mortality of pine forests following the Chornobyl disaster (Arkhipov et al., 1994). The progressive aspect of the shoot growth inhibition is consistent with a long-term (seed-to-seed) chronic laboratory irradiation study in *A. thaliana*, where irradiation at 2.3 mGy·h^{-1} starting at sowing resulted in significant shoot growth inhibition after 54 days of exposed growth, but not after 34 or 24 days (Vandenhove et al., 2010). In the present study, a 10-week irradiation at a dose rate of 682 μGy·h^{-1} (total dose of approximately 1 Gy) led to a 16 % inhibition of shoot growth (Fig. 1A). In comparison, a similar degree of growth inhibition (25 %) after irradiation of pine seedlings has been reported previously, but this required a much higher dose rate (100 mGy·h^{-1}) and total dose (14.4 Gy) when the exposure spanned just six days (Blagojevic et al., 2019a). Low-dose exposure may therefore be comparably effective at inducing phenotypic effects to shorter high-dose exposures if the exposure duration is long enough, possibly due to the accumulation of stress. Differences in developmental stage provide an alternative explanation, as radiosensitivity has been shown to vary with the age at exposure in *A. thaliana* (Biermans et al., 2015b). The significance of long-term versus short-term chronic irradiation has also been reported previously in *A. thaliana*, where a three-week exposure induced stronger shoot growth inhibition than shorter treatments despite lower dose rate (Goh et al., 2014).

The onset of shoot growth inhibition at week 6 coincided with a

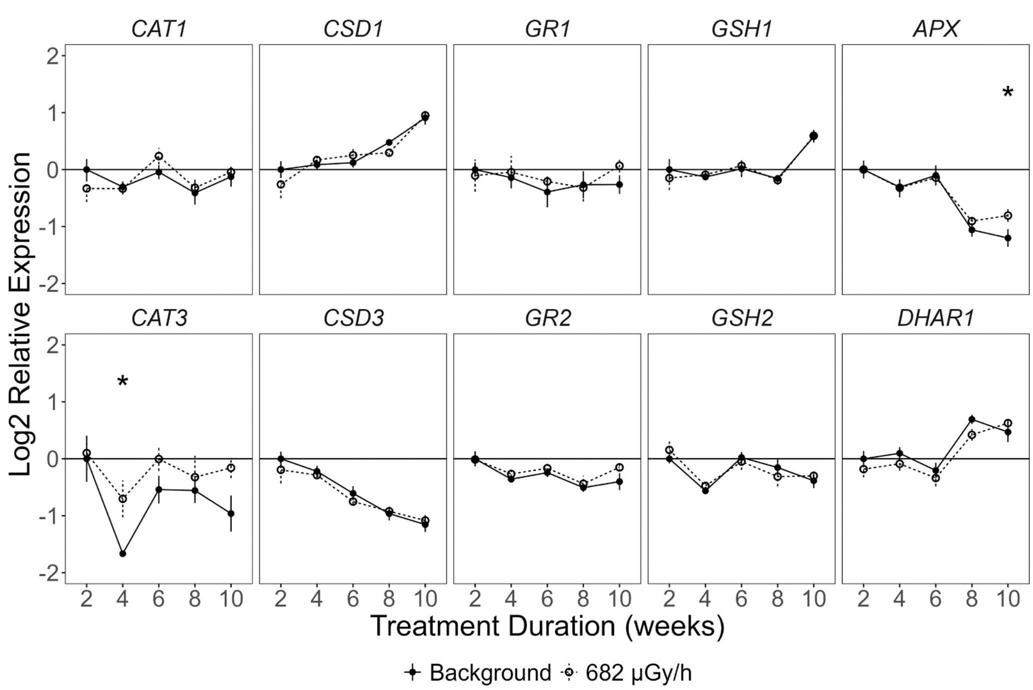

Fig. 5. Effects of chronic gamma irradiation on gene expression in pine seedling shoots. Log_2 relative expression values of transcripts involved in the oxidative stress metabolism in the shoots of pine seedlings continuously exposed to external gamma irradiation at 682 μGy·h^{-1} (open symbols, dotted lines) compared to controls exposed to normal background radiation (solid symbols, solid lines) over the course of 10 weeks. Expression was calculated relative to the mean of the control at the first timepoint. Mean data (n = 5) is presented with error bars indicating standard error. Significant differences between the two conditions are indicated as * ($p < 0.05$) using estimated marginal means and Tukey's correction for multiple comparisons. CAT1: CATALASE 1, CAT3: CATALASE 3, CSD1: COPPER/ZINC SUPEROXIDE DISMUTASE 1, CSD3: COPPER/ZINC SUPEROXIDE DISMUTASE 3, GR1: GLUTATHIONE REDUCTASE 1, GR2: GLUTATHIONE REDUCTASE 2, GSH1: GLUTAMATE-CYSTEINE LIGASE, GSH2: GLUTATHIONE SYNTHETASE, APX: ASCORBATE PEROXIDASE, DHAR1: DEHYDROASCROBATE REDUCTASE 1.

significant reduction in axillary bud formation (Fig. 2A), although this effect was transient. Furthermore, the number of outgrown branches at the end of the experiment was significantly lowered by irradiation (Fig. 2B), but whether this inhibition is also transient remains uncertain within the tested experimental timeframe. Interestingly, the relative sensitivity of pine shoot morphogenesis to radiation is a consistent finding. Despite variations in the specific alterations observed, this phenomenon has been encountered not only in this study but also in a previous laboratory exposure of pine seedlings (Blagojevic et al., 2019a) as well as in field research from radioactively contaminated areas like Chornobyl (Nybakken et al., 2023; Yoschenko et al., 2011) and Fukushima (Yoschenko et al., 2016). These developmental inhibitions are part of a wider suite of radiation-induced phenotypical changes reported in pine shoots, which includes altered needle morphogenesis (Geras'kin et al., 2021; Makarenko et al., 2016, 2021) and an increased number of needle resin ducts observed in radioactively contaminated areas (Nybakken et al., 2023). We must, however, acknowledge the complexities hindering comparison of laboratory findings to field conditions (Garnier-Laplace et al., 2013) such as the absence of internal emitters and beta-radiation in laboratory studies using external gamma irradiation. These additional exposure pathways contribute significantly to radiation dose rates in Chornobyl (Beresford et al., 2020), but are less prominent or negligible in the Fukushima case due to the fact that plants showed limited bio-accumulation of radiocesium, the predominant long-lived contaminant in the Fukushima Exclusion Zone (Horemans et al., 2018; Yoschenko et al., 2016).

4.2. Radiation-induced shoot growth inhibition coincides with an altered oxidative stress metabolism

Chronic irradiation of young pine seedlings had a considerable effect on the oxidative stress metabolism in shoot tissue, with the earliest evidence visible at the transcriptional level; *CAT3* was upregulated in irradiated seedlings after 4 weeks and remained slightly elevated throughout the experiment (Fig. 5). In line with the first part of our hypothesis, the onset of shoot growth inhibition and delayed bud initiation at week 6 coincided with a significant disruption of the seedlings' antioxidant system. Elevated GSSG without a corresponding rise in GSH at week 6 (Fig. 3A) led to increased glutathione oxidation (Fig. 3B), coinciding with significantly reduced activities of key antioxidant enzymes—glutathione reductase (GR), superoxide dismutase (SOD), and guaiacol peroxidase (GPOD; Fig. 4). Reduced GR activity may have hindered GSSG reduction, while lower SOD activity could have compromised superoxide detoxification, both potentially contributing to increased ROS accumulation and the glutathione oxidation status. These effects aligned temporally with the onset of shoot growth inhibition and delayed bud initiation (Figs. 1A, 2A), implicating radiogenic ROS production or the resulting oxidative stress response as potential contributors to the observed morphological changes. This notion is supported by previous research in pine seedlings, which indicated that radiation-induced shoot growth inhibition is associated with a significant increase in shoot H_2O_2 abundance, albeit at higher dose rates than the one tested here (Blagojevic et al., 2019b). The contribution of radiogenic ROS to altered pine shoot growth may also involve modulation of hormonal signals, as axillary bud initiation and outgrowth are governed by a complex regulatory network involving the plant hormones auxin and cytokinin, which are known to undergo intricate crosstalk with ROS signaling during stress conditions (Raja et al., 2017; Tognetti et al., 2017; Yang and Jiao, 2016; Yuan et al., 2024). Indeed, previous studies have linked radiation-induced alterations in pine shoot morphology to phytohormonal changes (Bitarishvili et al., 2024; Geras'kin et al., 2021).

It should be noted that, while the temporal alignment of the phenotypical and biochemical effects is striking, we cannot rule out possible involvement of radiation-induced DNA damage in the observed growth inhibition as we did not evaluate DNA damage following radiation exposure during this study. DNA damage is a hallmark of ionizing radiation exposure and seems to be a conserved phenomenon in chronically irradiated pine trees across exposures; both growth inhibition and shoot DNA damage correlated positively with dose rates ranging from 1 to 540 mGy h^{-1} in a 6-day gamma exposure of pine seedlings (Blagojevic et al., 2019a), while increased DNA damage could be observed in the shoots of Chornobyl pine trees exposed to dose rates as low as 11.1 $\mu Gy \cdot h^{-1}$ (Nybakken et al., 2023). These effects may stem from direct ionization of DNA by IR but are also mediated by radiogenic ROS, as it is well established that ROS exposure induces oxidative damage to DNA and leads to base modifications and single strand breaks (Roldán-Arjona and Ariza, 2009). Significant increases in shoot DNA damage were previously found to start occurring at lower dose rates compared to increasing shoot H_2O_2 abundance after chronic pine seedling irradiation, therefore indicating pine seedlings may be more sensitive to radiation-induced DNA damage than to radiation-induced oxidative stress (Blagojevic et al., 2019b). Thus, our observation of significant oxidative stress during irradiation treatment implies that considerable DNA damage could have occurred during the experiment. This may have had phenotypical implications, as aberrant pine phenotypes in Fukushima have previously been attributed to cytogenetic abnormalities resulting from DNA damage (Geras'kin et al., 2021; Vasiliev et al., 2020).

Despite clear biochemical shifts at the enzyme activity level (Fig. 4), gene expression of CSDs and GRs did not reflect these changes at the tested timepoints (Fig. 5), suggesting non-transcriptional regulation may be involved. Alternatively, these shifts may have been mediated by transcriptional responses of unmeasured transcripts. For example, expression of SOD isozymes such as CSD2, Fe-SOD, or Mn-SOD was not evaluated here but may have contributed to the observed decrease in SOD activity, while downregulation of glutathione peroxidase expression could have mediated the observed increase in GSH. It is also possible that transcriptional changes occurred in the relatively long two-week interval between measurements. Regulatory mechanisms such as a general downregulation of protein synthesis, a common stress response (Cramer et al., 2011), or increased protein turnover may also contribute to the observed enzyme activity reductions, as transcriptome analyses often indicate differential expression of genes involved in protein degradation and protein synthesis upon irradiation treatment in plants (Culligan et al., 2006; Gicquel et al., 2012; Ricaud et al., 2007; Van Hoeck et al., 2017). Further investigation is required to elucidate the regulatory layers modulating antioxidant enzyme activity under chronic radiation stress.

4.3. Towards acclimation through recovery of glutathione homeostasis

Following the initial oxidative disruption, seedlings demonstrated a remarkable capacity for recovery, supporting the second stage of our proposed mechanism. By week 8, a significant increase in reduced glutathione (GSH) was observed, which successfully restored the GSH:GSSG ratio to control levels (Fig. 3). This response appeared active and specific, as GSH levels showed opposite trends in irradiated versus control seedlings at this timepoint. Interestingly, this change in glutathione homeostasis correlated with a complete recovery of bud formation (Fig. 2A), implicating this swift glutathione-mediated alleviation of oxidative stress in the transient nature of the observed bud development inhibition. Increases in GSH abundance have also been reported in pine trees from the Chornobyl exclusion zone (Volkova et al., 2017), while other species such as herbaceous plants in the same area did not show this response (Volkova et al., 2021). *A. thaliana* also showed stable glutathione levels under chronic laboratory irradiation (Vandenhove et al., 2010; Vanhoudt et al., 2010). Nonetheless, increased GSH abundance as a radiation response is not pine-specific, having also been observed in chronically irradiated *L. minor* (Van Hoeck et al., 2015a; Xie et al., 2019). Interestingly, analysis of other antioxidative metabolites such as polyphenols and terpenoids in the needles of Chornobyl pine trees indicated an increased abundance of these compounds associated with highly radioactive areas, further highlighting the importance of antioxidative metabolites in the long-term radiation response of pines (Nybakken et al., 2023). A 6-day gamma irradiation treatment at dose rates up to 42.9 mGy h^{-1} yielded no significant changes to the abundance of phenolic antioxidants in the shoots of young pine seedlings while UV-B irradiation did increase this parameter (Blagojevic et al., 2019b). Hence, this class of antioxidative metabolites may be most relevant when the ionization intensity is limited. It could therefore be worthwhile to extend further investigations of the effect of chronic laboratory irradiation on antioxidant levels of pines into the realm of these compounds.

Despite clear shifts in glutathione levels, no transcriptional changes were detected in the biosynthetic genes *GSH1* and *GSH2* (Fig. 5), suggesting another regulatory mechanism is at play. Similar uncoupling between oxidative stress-induced GSH biosynthesis and GSH biosynthetic gene expression was reported in H_2O_2-treated *A. thaliana* (Xiang and Oliver, 1998). GSH synthesis is known to depend on precursor availability, sulphur assimilation, and post-translational enzyme modifications (Dorion et al., 2021). Notably, GSH1 activity can be enhanced under oxidative conditions via redox-sensitive disulfide bonding (Hicks et al., 2007; Yang et al., 2019), offering a possible mechanism for increased GSH synthesis in the absence of transcriptional induction. Additionally, transcriptomic analyses of long-established pine populations in Chornobyl suggest that glutathione levels may be altered in response to radiation exposure through changes to the metabolism of the glutathione precursor glutamine (Duarte et al., 2019). While our observation of limited transcriptional changes for key antioxidant genes (Fig. 5) appears to support the hypothesis that conifer radiosensitivity is linked to a limited transcriptional response (Bhattacharjee et al., 2023), the concurrent, dramatic shifts in glutathione oxidation and enzyme activities suggest the pines are still mounting a significant stress response. It may be that pines rely more heavily on non-transcriptional and metabolic regulation rather than wholesale transcriptional changes to initiate their response to chronic radiation stress, but why this type of response could be associated with increased radiosensitivity is unclear.

4.4. Distinct long-term survival strategies emerge

Our results support the final proposed stage of the response of pine trees to radiation: the transition towards a reconfigured, long-term acclimated state. By week 10, glutathione levels in irradiated shoots had returned to control values (Fig. 3), while GPOD and GR activities remained unchanged from week 6 onward (Fig. 4), implying that other defenses assume primary responsibility for the control of chronically increased radiogenic ROS levels. SOD, whose activity was significantly higher after ten weeks of irradiation (Fig. 4), represents a possible candidate. Because H_2O_2-scavenging enzymes such as GPX, APX, and CAT act downstream of SOD, sustained SOD upregulation would facilitate continuous dismutation of superoxide radicals into H_2O_2, which can then be further detoxified enzymatically or via non-enzymatic antioxidants. A similar biphasic SOD profile as the one observed here—early decline followed by late increase—was reported in *A. thaliana* under chronic γ-irradiation (Goh et al., 2014). Although comparable laboratory data for pines are lacking, related studies support long-term stress adaptation at the enzyme level: chronic UV exposure in Korean pine (*Pinus koraiensis*) elevated CAT, APX, and SOD activities (Zu et al., 2011), and field-grown pines near Chornobyl showed increased CAT activity and decreased POD activities at dose rates of 2.2 μGy·h^{-1} and 3.8–4.4 μGy·h^{-1} , respectively (Volkova et al., 2017). These observations suggest that long-term modulation of antioxidant enzyme activities—particularly sustained SOD activation—could constitute a survival strategy for managing radiogenic ROS once the glutathione pool has been re-equilibrated. Notably, this adaptation may be partially steered by natural developmental shifts in oxidative stress metabolism during seedling establishment. For instance, the inverse expression trends of *CSD1* and *CSD3* over the 10-week experiment suggest increasing cytosolic SOD importance, while concurrent upregulation of *DHAR* and downregulation of *APX* point to a shift in ascorbate recycling dynamics that may alter ROS buffering capacity (Fig. 5). The increased expression of *APX* after 10 weeks of irradiation further indicates that ascorbate may be an important antioxidant for coping with radiation stress at these longer time frames. This conclusion, however, must remain tentative, as an increase in *APX* transcripts cannot be definitively interpreted as an increase in ascorbate-based detoxification capacity without corresponding enzymatic activity and metabolite data. Further investigation into the role of the ascorbate metabolism in the radiation response of pine seedlings is required, e.g. through direct measurement of ascorbate levels and APX activity. Notably, the contribution of an altered ROS metabolism to the long-term radiation response of pines may even have a trans-generational component, as pine seedlings were found to have altered antioxidative capacities depending on whether they originated from trees growing in areas of Chornobyl with or without radioactive contamination (Rahman, 2021). However, whether the acclimation response observed in our 10-week study could contribute over longer timescales to (epi)genetic changes, as documented in irradiated pine populations (Bondarenko et al., 2023; Geras'kin and Volkova, 2014; Kovalchuk et al., 2003; Kuchma and Finkeldey, 2011; Volkova et al., 2018), and any resulting trans-generational effects is a critical area for future study.

5. Conclusion

In this study, we tested the hypothesis that the oxidative response of *P. sylvestris* seedlings to chronic low-dose irradiation is an active, multiphase process. Our findings support this mechanistic model, revealing a dynamic sequence of oxidative stress, recovery, and acclimation that underlies the observed impacts on shoot growth and development. A key observation was the dynamic modulation of glutathione metabolism in response to irradiation. An early phase of oxidative stress was indicated by elevated GSSG and an increased glutathione oxidation state at week 6, which correlated with reduced activities of crucial antioxidant enzymes (GPOD, GR, SOD) and the inhibition of shoot growth and axillary bud development. Importantly, after the initial response to enhanced radiation, this study revealed a subsequent recovery phase where GSH levels increased significantly by week 8, effectively normalizing the GSH:GSSG ratio. While the stable expression of genes involved in the glutathione metabolism indicates these changes may not be transcriptionally regulated, we cannot exclude the possibility of altered gene expression occurring in between measurements. By the experiment's end, a distinct antioxidant profile emerged, with SOD activity notably increased and *APX* expression upregulated in irradiated seedlings, suggesting a potential shift towards sustained enzymatic ROS scavenging and increasing dependence on ascorbate as a long-term coping mechanism. Taken together, these results highlight that while pine seedlings are sensitive to chronic irradiation, they also possess robust, temporally distinct biochemical mechanisms to manage radiation-induced oxidative stress, with glutathione playing a crucial, dynamically regulated role. Further research is needed to fully uncover the role of glutathione and ascorbic acid and their interplay with the antioxidative metabolism in plant radiation stress responses but should also investigate how these biochemical adjustments translate to long-term outcomes for pine tree health and population dynamics during radiation exposure. Understanding this link is critical for assessing the ecological impact of environmental radioactivity.

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CRediT authorship contribution statement

Brix De Rouck: Writing – original draft, Visualization, Investigation, Formal analysis, Conceptualization. **Esteban Suls:** Resources, Investigation. **Gustavo Turqueto Duarte:** Writing – review & editing, Supervision, Conceptualization. **Els Prinsen:** Writing – review & editing, Supervision. **Nele Horemans:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization.

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work the author(s) used Google Gemini 2.5 Pro in order to improve the readability and language of the manuscript. After using this tool/service, the authors reviewed and edited the content as needed and take full responsibility for the content of the published article.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.envexpbot.2025.106265.

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