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Characterizing dose response relationships: Chronic gamma radiation in Lemna minor induces oxidative stress and altered polyploidy level

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Abstract:

The biological effects and interactions of different radiation types in plants are still far from understood. Among different radiation types, external gamma radiation treatments have been mostly studied to assess the biological impact of radiation toxicity in organisms. Upon exposure of plants to gamma radiation, ionisation events can cause, either directly or indirectly, severe biological damage to DNA and other biomolecules. However, the biological responses and oxidative stress related mechanisms under chronic radiation conditions are poorly understood in plant systems. In the following study, it was questioned if the *Lemna minor* growth inhibition test is a suitable approach to also assess the radiotoxicity of this freshwater plant. Therefore, *L. minor* plants were continuously exposed for seven days to 12 different dose rate levels covering almost six orders of magnitude starting from 80 µGy h^{-1} up to 1.5 Gy h^{-1}. Subsequently, growth, antioxidative defence system and genomic responses of *L. minor* plants were evaluated. Although *L. minor* plants could survive the exposure treatment at environmental relevant exposure conditions, higher dose rate levels induced dose dependent growth inhibitions starting from approximately 27 mGy h^{-1}. A ten-percentage growth inhibition of frond area Effective Dose Rate (EDR_{10}) was estimated at 95 ± 7 mGy h^{-1}, followed by 153 ± 13 mGy h^{-1} and 169 ± 12 mGy h^{-1} on fresh weight and frond number, respectively. Up to a dose rate of approximately 5 mGy h^{-1}, antioxidative enzymes and metabolites remained unaffected in plants. A significant change in catalase enzyme activity was found at 27 mGy h^{-1} which was accompanied with significant increases of other antioxidative enzyme activities and shifts in ascorbate and glutathione content at higher dose rate levels, indicating an increase in oxidative stress in plants. Recent plant research hypothesized that environmental genotoxic stress conditions can induce endoreduplication events. Here an increase in ploidy level was observed at the highest tested dose rate. In conclusion, the results revealed that in plants several mechanisms and pathways interplay to cope with radiation induced stress.

1. Introduction

Although the environment is continuously exposed to natural ionising radiation of cosmic and terrestrial origin, high levels of ionising radiation could potentially lead to disturbances in population ecosystems. Enhanced radiation levels can be the result of controlled anthropogenic activities from naturally occurring radioactive materials (NORM) industry, nuclear accidents or nuclear power production. During the last decade, international organisations like International Atomic Energy Agency (IAEA) and International Commission on Radiological Protection (ICRP) supported the development of guidance for environmental risk assessment and ecological protection criteria (IAEA, 1992; ICRP, 2003). In the framework of the European funded project ERICA[1], an effect database was established which holds data on radiation effect observations for non-human biota (Copplestone et al., 2008). From this database, it was clear that most of the studies performed so far deal with responses to acute, high doses rather than more environmentally relevant low dose and chronic exposure conditions (Garnier-Laplace et al., 2004; Esnault et al., 2010). Hence, more biological effect data in chronic, low-dose exposure conditions and on key wildlife groups are required as stated in the strategic research agenda of the ALLIANCE [2] (Hinton et al., 2013).

Gamma emitters and other radionuclides are routinely released into aquatic environments from nuclear power plants and nuclear fuel reprocessing plants. However, the possible biological responses on aquatic organisms and plants in particular, are still far from understood. Aquatic plants are defined as plants whose photosynthetic active parts are permanently or semi-permanently submerged in the water or float on the surface, and thereby play a vital role in healthy ecosystems. In terrestrial plants, both lab and field studies have shown that high external gamma radiation can affect morphology, physiology and reproductive capacity (Daly and Thompson, 1975; Sheppard et al., 1992; Kovalchuk et al., 2000; Kim et al., 2005; Wi et al., 2007). However, irradiation experiments have up to now used rather different plant species and exposure conditions complicating comparative conclusions. Despite these different approaches, literature have pointed out that plant species, plant organs and plant developmental stage show significant differences in radiosensitivity (Sparrow and Miksche, 1961; Kawai and Inoshita, 1965; Killion and Constantin, 1972; Daly and Thompson, 1975; Kovalchuk et al., 2000; Wi et al., 2007). Additionally, depending on the duration and level of the radiation exposure applied, plants showed dissimilarities in gene expression profiles depending on whether plants were acutely or chronically irradiated (Kovalchuk et al., 2000; Kovalchuk et al., 2007). In *Arabidopsis*, oxidative stress related genes seemed to be the most represented group in responding to both radiation conditions (Kovalchuk et al., 2007; Gicquel et al., 2012).

[1] Environmental Risk from Ionising Contaminants: Assessment and Management
[2] The European Radioecology Alliance (www.er-alliance.org)

Healthy growing plants continuously generate low concentrations of Reactive Oxygen Species (ROS) as by-products of aerobic and anaerobic metabolic pathways in the chloroplasts, mitochondria and peroxisomes. In response to abiotic stress however, ROS can accumulate in plant cells, resulting in oxidative stress possibly leading to severe biological damage (Apel and Hirt, 2004; Gill and Tuteja, 2010). In radiation-exposed plant cells, ROS accumulation is additionally increased among others through the radiolysis of H_2O possibly leading to oxidative stress (Von Sonntag, 1987), although it has been confirmed that the production of ROS by radiolysis is negligible under environmental relevant exposure conditions (Smith et al., 2012). To counteract this ROS induced oxidative stress, plants can modulate antioxidative defence ROS-scavenging enzymes and metabolites (Gill and Tuteja, 2010). Different antioxidative enzymes like catalase (CAT), glutathione reductase (GR), superoxide dismutase (SOD), ascorbate peroxidase (APOD), syringaldizyne peroxidase (SPOD) and guaiacol peroxidase (GPOD) and metabolites like glutathione, flavonoids, phenolic compounds and carotenoids are typically induced in order to reduce increased concentrations of singlet oxygen ($^1O_2$), superoxide radical (O_2^-), hydrogen peroxide (H_2O_2) or hydroxyl radical ($HO^\bullet$) in plant cells (Apel and Hirt, 2004). These antioxidative defence systems have their specific reactants, exhibiting different reaction kinetics and locations in subcellular components. For instance, CAT can independently convert H_2O_2 to H_2O and O_2 whereas APOD requires the oxidation of its co-substrate ascorbate to reduce H_2O_2 (Mittler et al., 2011). The metabolites ascorbate (ASC) and glutathione (GSH) are key players in the ASC-GSH cycle, forming an important mechanism of the antioxidative defence system (Noctor and Foyer, 1998).

Plants exposed to gamma radiation face deleterious effects that are either directly or indirectly induced through oxidative stress. The highly reactive ROS species, in particular $HO^\bullet$, can readily target DNA by the oxidation of nucleic acids (Balasubramanian et al., 1998). Additionally, ionising radiation can directly cause DNA lesions by deposition of energy leading to excitations and ionisation events, which may induce a spectrum of rearrangements and modifications to chromosomes. DNA damage affected on only one strand, like single strand breaks or single base conversion, can be repaired relatively easily by the presence of the complementary strand as a template. Hence when double strand breaks are being induced this is presumed to be more severe leading to more detrimental damage (Tuteja et al., 2001). As plants require light for photosynthesis their chloroplasts continuously generate ROS, and hence plants are believed to be constantly exposed to DNA damaging agents (Landry et al., 1995). Therefore plants require a DNA repair machinery of high efficiency and fidelity to protect DNA integrity. As such, when a DNA error is detected by DNA checkpoints, cell cycle progression can be delayed or arrested to gain time for repairing the damage (Britt, 1996). Cyclin-dependent kinases (CDKs) are mitotic regulators that guide two major phases during the cell division cycle. The genome is replicated during the synthesis phase (S-phase) and is afterwards halved during the final step of the mitosis cell cycle (M-phase) (De Schutter et al., 2007).

Transition to the latter can only happen if DNA repair has occurred, although, under specific circumstances it is already observed that S-phase can still proceed without subsequent chromosome separation and cytokinesis ending in polyploid cells (De Veylder et al., 2011). This process, called endoreduplication, is well known in a variety of structural plants tissues to achieve growth by cell expansion. However, emerging data in plant research suggest another conserved role for endoreduplication in response to genomic instability caused by genotoxic environmental stressors (De Veylder et al., 2011).

Current literature indicated that for freshwater ecosystems interpretation of radioecological consequences is limited due to a lack in aquatic plant representatives (Garnier-Laplace et al., 2006; Garnier-Laplace et al., 2013). *L. minor* is a free-floating freshwater macrophyte for which OECD guidelines have been published to test the environmental toxicity of chemical contaminants in higher aquatic plants (OECD, 2006; Areum Park, 2013). In this study, it was questioned if this *L. minor* growth inhibition test is a suitable approach to also assess the radiotoxicity of this freshwater plant. As such the sensitivity of *L. minor* to gamma radiation was first analysed by evaluating the growth inhibition (as described in the OECD guidelines) in the plants exposed to dose rates ranging from background level to 1500 mGy h^{-1}. As radiation induced ROS might be key players in the induction of adverse growth effects, the antioxidative response was further studied in the exposed *L. minor* plants. Additionally, DNA ploidy of *L. minor* plant cells was analysed since it is hypothesized that genotoxic stressors induce endoreduplication events in plants (De Veylder et al., 2011). Linking growth effects of irradiated plants with their respective physiological and genomic transformations, will allow to better understand underlying mechanisms of how chronic induced radiation stress influences plant systems.

2. Material and Methods

2.1. Culture stock

Lemna minor cv. Blarney plants (Serial number 1007, ID number 5500) were obtained from Dr M. Jansen (University College Cork, Ireland) and aseptically cultured in a growth chamber in 250 ml glass Erlenmeyer flasks containing half-strength Hütner medium (Brain and Solomon, 2007) under continuous light (Osram 400 W HQI-BT daylight, OSRAM GmbH, Augsburg, Germany, 102 ± 1 µmol m^{-2} s^{-1}) at 24.0 ± 0.5 °C. Plants were subcultured every 10-12 days by transferring three plants to 100 ml of fresh growth medium. To obtain a sufficient quantity of plants and a homogenous plant population, 1 week prior to an experiment five mature plants (3-4 fronds) were transferred to 100 ml of fresh medium.

2.2. Gamma irradiation

Plants were chosen randomly from at least four pots of precultured plants. Experimental conditions followed guideline 221 of the OECD (OECD, 2006) as close as possible with a modified growth medium containing (in mg l^{-1}) 889 KNO_3, 944 $Ca(NO_3)_2.4H_2O$, 500 $MgSO_4.7H_2O$, 9 Na-EDTA, 3 tartaric acid, 1,86 H_3BO_3, 0.22 $ZnSO_4.7H_2O$, 0.12 $Na_2MoO_4.2H_2O$, 0.08 $CuSO_4.5H2O$,3,62 $MnCl_2.4H2O$, 5.4 $FeCl_36H_2O$ (Maeng and Khudairi, 1973) with lower KH_2PO_4 concentrations (0.5 mg l^{-1}). To stabilise pH during toxicity tests, 5 mM filter-sterilised (0.22 µm) MES [2-(N-morpholino) ethanesulfonic acid] was added. The pH of the medium was adjusted to 5.5 by addition of filter-sterilised (0.22 µm) NaOH or HCl from a 1 M stock solution.

Precultured plants were transferred to the irradiation facility at SCK•CEN, where they were kept under conditions similar to those in the growth chamber (control). Two experiments were performed to obtain a broad range of radiation dose rates. In the low dose rate experiment, the plants were exposed to a ^{137}Cs gamma source (1.30E+11 Bq) for 9817 minutes covering a dose rate interval between 0.08 mGy h^{-1} and 4.95 mGy h^{-1}. The plants from high dose rate experiment were exposed for 9824 minutes using a ^{137}Cs gamma source (1.25E+12 Bq) resulting in dose rate levels from 27 mGy h^{-1} to 1500 mGy h^{-1}. The exposure treatment was every day aborted for ±30 minutes (time not counted in total exposure time) to allow for taking pictures of the *L. minor* fronds. The total doses were obtained through thermoluminescent dosimetry. Control plants were kept in a separate growth chamber with conditions closely resembling environmental conditions for each experiment.

2.3. Plant growth

Growth parameters, in terms of relative frond number, relative frond area and fresh weight were analysed according to OECD guideline 221 (OECD, 2006). Images for determination of average specific growth rate and average specific frond area were every day taken, and analysed with ImageJ open source software

(version 1.43) (Abràmoff et al., 2004). Fresh weight was determined by weighing all collected plant material from individual pots after quickly patting dry with clean tissue. Plants were afterwards snap frozen in liquid nitrogen for storage at -80°C for biochemical analysis. The average specific growth rate for the endpoint considered was calculated according to the OECD guidelines 221 (OECD, 2006). Percentage inhibition of growth rate was then calculated for each test concentration to construct the dose response curves. The doubling time for frond number in non-irradiated controls was 2.13 ± 0.09 days and 2.40 ± 0.07 days for the low and high dose rate experiments respectively, achieving the validity criterion of the ecotox test which is a 2.5 days doubling time. All test solutions pH remained constant at 5.5 ± 0.5. There were 9 replicates for each dose rate condition and control.

2.4. Antioxidative enzyme activities

Frozen plant tissue (50-80 mg FW) was homogenized under frozen conditions using liquid nitrogen with two tungsten carbide beads (Qiagen) of 3-mm diameter in a Retsch Mixer Mill MM400 at 30 Hz for 3 min after a spatula of insoluble polyvinylpyrrolidone (PVP) was added. Hereafter, 400 µl ice-cold 0.1 M Tris-HCl buffer (pH 7.8) containing 1 mM EDTA and 1 mM dithiothreitol was added to the frozen homogenised tissue, the mixture was vortexed and subsequently centrifuged at 20,000 x g at 4 °C for 10 min. The supernatant was kept on ice and used fresh for determination of enzyme capacities. All measurements were performed at room temperature (20–22 °C) based on a spectrophotometric assays as previously described (Horemans et al., 2015). At least 3 replicates were analysed in triplicate. Statistical analysis was performed on the measured data before expressing them relative to controls.

2.5. Metabolite measurements

Oxidized and reduced forms of ascorbate (ASC) and glutathione (GSH) were measured spectrophotometrically using a plate-reader assay (PowerWave HT Microplate Spectrophotometer, Biotek). Frozen plant tissue (50–80 mg FW) was homogenised under frozen conditions using two tungsten carbide beads (Qiagen) of 3-mm diameter in a Retsch Mixer Mill MM400 at 30 Hz for 3 min. Measurements were done as described by (Queval and Noctor, 2007) following modifications as described in Horemans et al. (2015). Redox status is expressed as ASC/ASC+DHA*100. At least 3 replicates were analysed in triplicate. Statistical analysis was performed on the measured data before expressing them relative to controls.

2.6. Flow cytometric analysis

The determination of DNA content was performed on fresh plant material immediately after harvesting using the Cystain PI Absolute P kit (Partec). *L. minor* fronds were separately chopped with a fresh razor blade in a petri dish containing 500 µl extraction buffer. After 1 minute incubation, the solution was filtered through a 50 µm nylon filter (Celltrics) and 2 mL staining solution, consisting of 2 mL staining buffer, 120 µL propidium iodide (PI) solution and 6 µL RNase per sample, was added to the flow-through. The samples were then incubated in the dark for at least one hour and their nuclear DNA content was analysed on the BD Accuri C6 Flow Cytometer (BD Biosciences) with a FL2 585/40 nm filter. At least 6 fronds were analysed for each exposure condition.

2.7. Statistical analysis

All data have been presented as mean values ± standard error (SE). Statistical analysis was performed with the open-source software package R (R i386 2.15.5; R Foundation for Statistical. Computing, Vienna, Austria). Normal distribution was tested with a Shapiro-Wilk test. A Barlett's test was used to test for homoscedasticity. To identify statistical differences between treatments, a one-way ANOVA was performed. When significant differences (p-value < 0.05) were found, a Tukey post hoc test was applied to further discriminate between significantly different groups. Student's unpaired two-tailed t-test was used for single comparisons. The dose response curves were modelled using the three parameter log-logistic drm equation from the drc package available in the software package R (p-value < 0.05) (Ritz and Streibig, 2005).

3. Results

3.1. Growth inhibition on different endpoints

In order to study the impact of chronic gamma radiation in *L. minor*, plants were continuously exposed to different levels of radiation for seven days. Two single exposure experiments were performed to obtain a broad range of radiation dose rates. The low dose rate experiment covered a dose rate interval between 0.08 mGy h^{-1} and 4.95 mGy h^{-1} while the high dose rate experiment exposed plants from 27 mGy h^{-1} up to 1500 mGy h^{-1}. After seven days of exposure, the results revealed no observable growth effects on the individual level in the low dose rate experiment. However, exposing plants to higher dose rates induced significant relative growth inhibition for all endpoints measured starting from a dose rate of 120 mGy h^{-1} (figure 1). Only growth inhibition calculated on frond area was already significantly different at a dose rate of 27 mGy h^{-1} making frond area as the most sensitive endpoint for *L. minor* growth. At the highest tested dose rate (1500 mGy h^{-1}), growth was reduced for 54 ± 4% on fresh weight, 62 ± 3% on frond area and 65 ± 5% based on frond number (figure 1).

Figure 1

The growth responses obtained from both experiments were fitted using log-logistic models at which maximal growth reduction was fixed to 100%. The slope and inflection parameters were -0.9 and 877 for frond area, -1.1 and 931 for frond number and -1.2 and 1278 for fresh weight in the equation model. Subsequently, the model was used to assess the Effective Dose Rate (EDR) values for all endpoints (table 1). The EDR_{10}-value for frond area gave an estimated dose rate of 95 ± 7 mGy h^{-1}, followed by 154 ± 15 mGy h^{-1} and 152 ± 13 mGy h^{-1} for fresh weight and frond number, respectively. These EDR_{10} values and the EDR_{30} values were significantly different for the three growth endpoints (table 1). On the contrary, EDR_{50} values did not differ significantly between growth endpoints (p-value < 0.05, one-way ANOVA).

Table 1: Effective Dose Rate (EDR) values (± SE) for different growth endpoints for *L. minor* plants exposed to gamma radation for 7 days. An asterix shows a significance difference of $p < 0.05$.

Endpoint	EDR_{10}	EDR_{30}	EDR_{50}
Frond Area	*95 ± 7 mGy h^{-1}	*366 ± 15 mGy h^{-1}	852 ± 41 mGy h^{-1}
Frond Number	169 ± 12 mGy h^{-1}	477 ± 19 mGy h^{-1}	915 ± 36 mGy h^{-1}
Fresh Weight	153 ± 13 mGy h^{-1}	556 ± 26 mGy h^{-1}	1250 ± 76 mGy h^{-1}

Figure 2

The average specific growth of *L. minor* throughout the tested time period was performed for each dose rate condition based on image analysis of frond area. No differences in growth were observed in the low dose rate experiment when considering the growth rate responses for each dose rate condition on a daily basis. However, in the high dose rate experiment, a significant growth arrest was observed at the two highest dose rates (figure 2). The observed growth arrest started already after three days of exposure in plants exposed to 1500 mGy h^{-1} (p-value < 0.05, one-way ANOVA), but no indications of necrosis or cell breakdown were observed at the end of the experiment. As stated before, all plants from high dose rate experiment showed a significant growth reduction for frond area at the end of the experiment compared to control.

3.2. Antioxidative defence system

Figure 3

To characterize dose-dependent antioxidative stress responses in continuously gamma-exposed *L. minor* plants, enzyme activities in the antioxidative pathways including catalase (CAT), glutathione reductase (GR), superoxide dismutase (SOD), ascorbate peroxidase (APOD), guaiacol peroxidase (GPOD) and syringaldizyne peroxidase (SPOD) were analysed (figure 3). Only enzyme activities of the high dose rate experiment are presented since plants from the low dose rate experiment showed no significant radiation-induced responses in antioxidative enzymes or metabolite levels. Measurements were restricted to a maximum dose rate of 423 mGy h^{-1} as higher dose rates resulted in a too small biomass to allow for analysis. Measured antioxidative enzyme activities after seven days exposure at different dose rates ranging from 27 mGy h^{-1} to 423 mGy h^{-1} are presented in figure 3. Except for CAT, no significant differences in enzyme activities were found in plants exposed to the lowest dose rate of 27 mGy h^{-1} (p-

value < 0.05, one-way ANOVA). These enzyme activities remained approximately unaltered up to a dose rate of 62 mGy h^{-1}. At 120 mGy h^{-1} and 232 mGy h^{-1}, enzyme activities of CAT, GPOD, SPOD and SOD were significantly enhanced. Ultimately, the enzyme activities for CAT and APX peaked at the highest dose rate evaluated (423 mGy h^{-1}). At this dose rate, an approximately twofold increase in activity was observed for these enzymes. The enzyme GR was the only exception exhibiting a transient decrease in activity with increasing dose rate, but showed increased activity at the highest dose rate.

Figure 4

Ascorbate (ASC) and glutathione (GSH) concentrations were measured to evaluate their contribution in the antioxidative defence system (figure 4). Additionally, oxidized and reduced metabolites were determined to study their redox status. No shift in redox status was observed at any dose rate intensity for GSH. In contrast, increasing dose rates caused a decreasing trend in redox status for ASC and was found to be significantly lower at the highest dose rate tested (232 mGy h^{-1}). At this dose rate, the redox status switched to 57 ± 2% while control plants exhibited a redox status of 78 ± 4%. Total GSH and total ASC concentrations remained unaffected for lower dose rates. At 232 mGy h^{-1}, a significant increase in total concentrations of both antioxidants was found.

3.3. Endoreduplication

Figure 5

DNA content of *Lemna* nuclei was determined in order to examine possible effects on ploidy level after 7-day chronic gamma radiation treatment from 27 mGy h^{-1} up to 1500 mGy h^{-1}. The relative nuclear DNA contents from exposed plant cells, given as the average percentage ploidy level of the whole plant, are shown in figure 5. Only diploid and tetraploid cells were detected in control plants with approximately five times more diploid compared to tetraploid nuclei under controlled conditions. This ratio remained unaffected at exposures treatments up to 423 mGy h^{-1}, but at a dose rate of 1500 mGy h^{-1}, a significant increase in tetraploid nuclei was observed (p-value < 0.05, one-way ANOVA).

4. Discussion

The aim of this study was to evaluate chronically induced radiation stress responses in *Lemna minor* plants. As no information on the radiation levels inducing growth effects in *L. minor* was available, plants were first exposed for seven days to 12 different dose rate levels covering almost six orders of magnitude starting from 80 μGy h^{-1} up to 1.5 Gy h^{-1}.

The present study assessed the hypothesis that the standardized duckweed test is suitable to evaluate the toxicity of aquatic plants to external gamma radiation. Taking into account the plants used to start the experiment, 85% of the total *L. minor* plant population were continuously exposed until harvest. As such, the exposure treatment was regarded as a chronic irradiation treatment of *L. minor* plants. Screening values of 10 μGy h^{-1} and 400 μGy h^{-1}, respectively were previously set for plant species (Howard et al., 2010). These screening values can be used to screen out sites of no concern *i.e.* sites were ecosystem is not at risk. For aquatic organisms, these screening values are close to the estimated dose rate levels in highly contaminated sites. For example exposure dose rates of seaweed and algae were estimated to 100 μGy h^{-1} and 633 μGy h-1, respectively, one month after the Fukushima accident (Vives I Battle et al., 2014), and aquatic plants from the southern Urals received a dose rate of 450 μGy h^{-1} after the Mayak accident (Kryshev et al., 1997). As significant growth inhibition on *L. minor* was only observed at dose rates 2 orders of magnitude higher then to the highest environmental dose rate levels estimated so far, it can be suggested that *L. minor* growth inhibition test is not appropriate to analyse environmental exposure situations.

In contrast to environmental relevant dose rate levels, it was shown that higher levels of gamma radiation had a negative impact on *L. minor* growth. Growth was assessed on three different growth related endpoints: frond area, frond number and fresh weight. Comparing the EDR_{10} and EDR_{30} values of the different endpoints indicated that frond area was the most sensitive growth related endpoint. The EDR_{10} values were 95 ± 7 mGy h^{-1}, 154 ± 15 mGy h^{-1} and 152 ± 13 mGy h^{-1} for frond area, frond number and fresh weight respectively. Garnier- Laplace et al. (2013) described the statistical distribution of EDR_{10} values for terrestrial plants of chronically exposed to gamma radiation under laboratory and field conditions considering endpoints directly relevant to population demography. However, from all available literature, only nine terrestrial and no aquatic plant studies reached the selection criteria to be included in this SSD. This points the lack of high quality data on aquatic plant species as also indicated by Garnier-Laplace et al. (2006). The most radioresistant plant species included in the SSD curve was *Horedeum sp* with an estimated EDR_{10} value of 70 mGy h^{-1}. This implies that the EDR_{10} values on growth related endpoints obtained in the present study for *L. minor* which range from 95 to 154 mGy h^{-1} are among the higher dose rates when compared to existing SSD for terrestrial plants.

However, it is anyhow difficult to compare results from different studies since it is known that plant species, organelles and development stage influence the extent of the radiation-induced effects (Wi et al. 2005; Kovalchuk et al. 2007; Vanhoudt et al., 2010; Biermans et al. 2015). Differences in sensitivity were also found in *Daphnia sp.*, another aquatic organism that is specified to be used in the OECD Guidelines. Exposing *Daphnia magna* resulted in an EDR_{10} value of 16.8 mGy h^{-1} whereas *Daphnia pulex* showed an EDR_{10} value of 277 mGy h^{-1} (Marshall, 1962; Gilbin et al., 2008). Adam-Guillermin et al. (2012) reviewed published effect data on gamma irradiated aquatic invertebrates and vertebrates and concluded that the latter are overall more radioresistant, although all aquatic vertebrates showed a more sensitive radiation effect than *Daphnia pulex*. Concisely, the EDR_{10} values obtained for growth inhibition of *L. minor* lay within the range of the more radioresistant aquatic organisms.

In addition to the effect of radiation on *L. minor* on growth related endpoints, some ROS scavenging biosynthetic pathways involving both enzymatic and non-enzymatic processes were studied. To date, only a few studies investigated these physiological and biochemical responses in chronically irradiated *A. thaliana* (Vandenhove et al., 2010) and *Stipa capillate* plants (Zaka et al., 2002) and only at radiation levels at which plant growth was not or only weakly affected. Generally these two studies revealed minor dose related responses in antioxidative enzyme activities and no enhanced lipid peroxidation or DNA strand breakage could be demonstrated. In present study, five out of six of the studied antioxidative enzymes showed dose-dependent increases in activities. Based on the observed data, these responses of the ROS defence system could be divided in different stadia; CAT activities showed the most sensitive response as it was already increased at dose rate levels below the EDR_{10} value for growth inhibition, whereas CAT, SOD, GPOD and SPOD activities were enhanced between the EDR_{10} and EDR_{30} values. Ultimately, the key players of the ascorbate-glutathione cycle showed strong alterations at the two highest dose rates tested. Since CATs are the principal H_2O_2-scavenging enzymes in plants, they are considered to be a cellular sink for H_2O_2 (Mittler et al., 2004). No differences in antioxidative enzyme activities were observed in *L. minor* plants exposed to dose rates from 0.08 mGy h^{-1} up to 4.95 mGy h^{-1} which is in agreement with the findings of Smith et al. (2012) where no additional ROS formation was expected to occur at these low dose rate levels.

Catalase activity was already increased at the lowest irradiation level in the high dose rate experiment. Sensitive responses on CAT activity were also reported in a 124-day experiment at 65 µGy h^{-1} with *S. capillata* (Zaka et al., 2002) and an increased expression level of the CAT1 and CAT3 genes were observed in a 3-day exposure treatment at 50 mGy h^{-1} in *A. thaliana* (Vanhoudt et al., 2010). In contrast, during chemical or metal induced toxicity in *L. minor* plants catalase is only induced at higher toxicity levels and often after other antioxidative enzymes like SOD or POD have already been elevated. Uranium

and cadmium triggered CAT activity when a 20 % growth inhibition was induced in *L. minor* (Horemans et al., 2015), while zinc did not affect CAT activity in *L. minor* plants at a level that induced 35 % growth inhibition (Radic et al., 2010). Compared to metals like uranium, cadmium and zinc, gamma irradiation could result in a more scattered H_2O_2 production. In this way, CAT could be responsible to maintain H_2O_2 levels within acceptable physiological concentrations. This could suggest that CAT might play a sensitive role in the antioxidative response of plants chronically exposed to gamma radiation stress compared to other abiotic stress stimuli. Between 120 mGy h^{-1} and 232 mGy h^{-1}, increased enzyme activities of CAT, SOD, GPOD and SPOD were observed. Despite the fact that no ROS species were measured in *L. minor* plants, it could be concluded that increased concentrations of ROS were present in *L. minor* plants exposed at these dose rates. At the next dose rate tested, 423 mGy h^{-1}, GR was reactivated and almost a twofold increase in APX activity was observed. APX utilizes ASC as electron donor to reduce H_2O_2 into water, whereas GR converts oxidized glutathione (GSSG) to GSH using NAD(P)H as an electron donor. Both enzymes are key players within the ASC-GSH cycle, which is an important component of the ROS scavenging system in plants (Noctor and Foyer, 1998). Under healthy growth conditions, NAD(P)H is mainly used as reducing power for the biosynthetic reactions in the Calvin cycle to assimilate carbon dioxide. Therefore, the ASC-GSH cycle is tightly regulated and is only activated when the redox balance is hampered. Although metabolite concentrations of ASC and GSH could, due to limited plant material, not be measured at this dose rate, strong increases on the antioxidants were observed at the second highest dose rate. First, increased concentrations of ASC and GSH resulted in a higher capacity to counterattack H_2O_2 -molecules and secondly, a shift in redox status of ASC indicates that the cellular redox balance within the cells was disturbed. Based on these observations, it is likely that ROS production in chronically gamma-irradiated plants can be kept under control up to high dose rates by a tight regulation and simultaneous participation of different ROS scavenging enzymes and metabolites.

Growth arrest was observed after three days of exposure at the highest dose rate suggesting severe biological damage at high radiation conditions. Both ionising radiation and ROS can attack DNA leading to severe DNA damage like single strand and double strand breaks. Nonetheless, all *L. minor* plants had a healthy phenotype at the end of the experiment as no changes in frond anatomy were visually observed. In support of this, photosynthetic pigments chlorophyll a, chlorophyll b and carotenoids concentrations in *L. minor* fronds remained unaltered at dose rates up to 450 mGy h^{-1} under similar growth conditions (Horemans, personal communication). A mitotic growth arrest can be regulated by cell-cycle checkpoints (CDKs) which are stimulated under DNA damaging conditions to activate DNA repair systems in order to restore genomic strands before proceeding to the final steps in mitosis cycle and can lead to endoreduplication in plants (Kirik et al., 2007). For example, the growth arrest observed in UV-exposed epidermal cells of cucumber trichomes was accompanied by an increased ploidy level (Yamasaki et al.,

2010). Another study illustrated that the treatment of plants with DNA damaging agents resulted in enhanced ploidy levels, emphasizing that DNA damage can indeed trigger endoreduplication (Ramirez-Parra and Gutierrez, 2007). In agreement with these studies, *L. minor* plants showed a significant increase in ploidy level although this was only found at 1500 mGy h^{-1}. These data support the hypothesis that plants allow endoreduplication events when they are insufficient in maintaining their genomic stability (De Veylder et al., 2011). Additionally, it has been shown that oxidative stress checkpoints can also block mitosis in plants indicating that different stress conditions regulate plant cell cycle progression (Reichheld et al., 1999). Whether oxidative stress or DNA damage was responsible for the observed growth arrest in *L. minor* could not be elucidated in this study. Consequently, the underlying mechanisms on how endoreduplication contributes to plant growth under extreme physiological conditions are still far from understood.

5. Conclusion

In conclusion, our data demonstrated for the first time that chronic gamma irradiation provokes dose-dependent developmental, morphologic and biochemical changes in *L. minor*. However, these changes were observed at dose rate levels exceeding environmental relevant exposure conditions. High levels of ionising radiation induced a growth inhibition of more than 50% after seven days on all measured individual growth endpoints. As plants were continuously exposed and new plants were formed during the test, it is almost impossible to verify at which level plants start to sense ionising radiation as a serious threat. According to the observed EDR_{10} for growth inhibition of the present study, it can be concluded that radiosensitivity of *L. minor* is low in comparison with terrestrial plants and other aquatic organisms. Also, the antioxidative defence system, with CAT as most sensitive enzyme, was stimulated below to this dose rate level and was enhanced upon increasing chronic radiation levels. These dose dependent shifts in antioxidative defence system indicate that *L. minor* can strictly coordinate ROS scavenging enzymes and metabolites activities upon increasing radiation induced stress. In conclusion, it has been shown that in *L. minor* several mechanisms and pathways interplay to cope with radiation induced stress. In future studies, studies on molecular level need to be examined to support the observed biochemical changes and further help to unravel and understand the biological complexity of plant stress response to ionising radiation.

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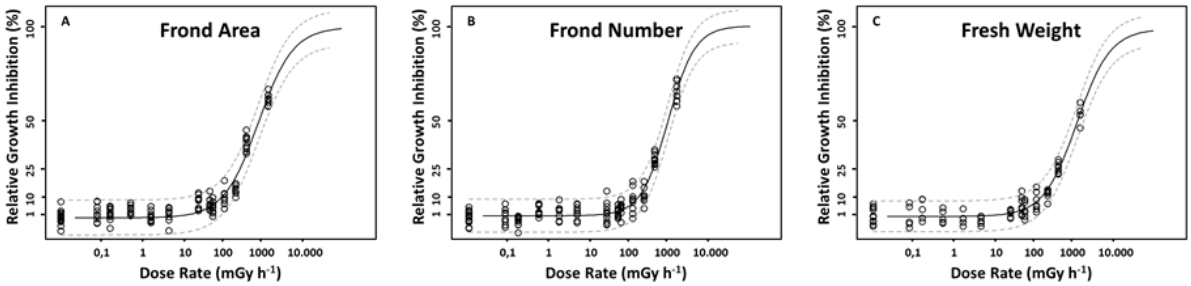

Figure 1: Dose response curve of *L. minor* plants exposed for 7 days to gamma radation. The dose response curves are presented as relative growth inhibition based on frond area (A), frond number (B) and fresh weight (C). In addition to the individiual points, the solid line shows the log-logistic function fitted through the data and the dashed lines indicate the 95% prediction interval.

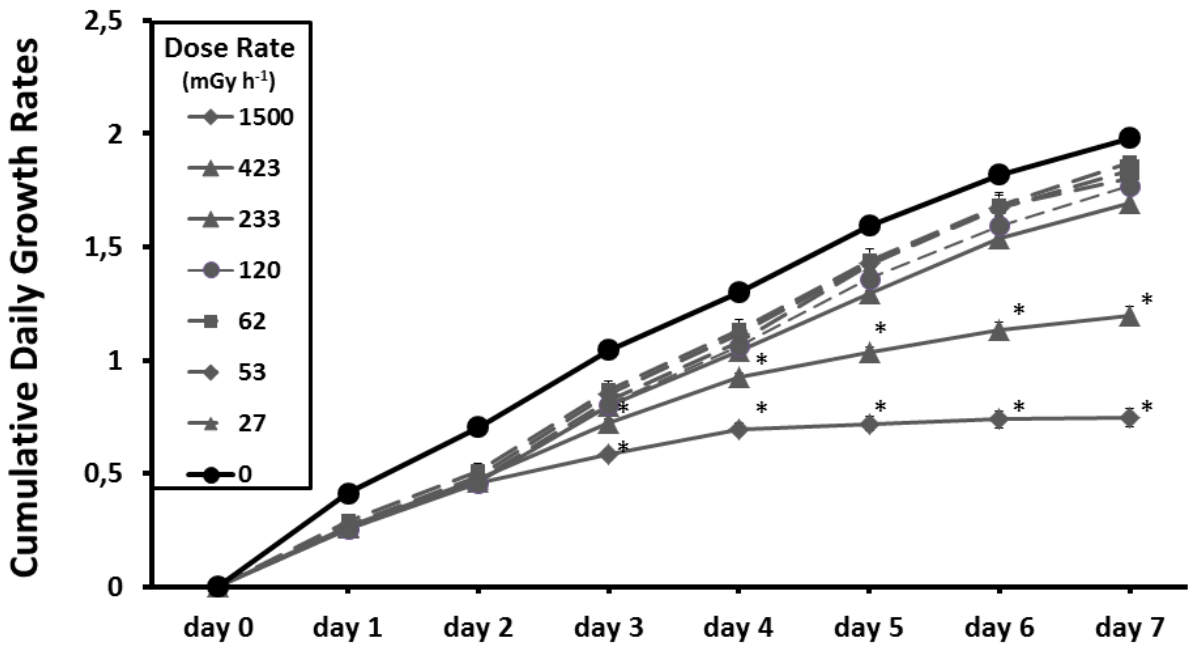

Figure 2: The average specific growth of L. minor on frond area exposed to different dose rates levels of gamma radation for 7 days. Different dose rate conditions are indicated on the left side. Asterixes indicate significant differences for plant growth compared with growth rates from any other dose rate condition. Image analyses was performed on 9 biological replicates for each dose rate condition and are represented as mean ± SE.

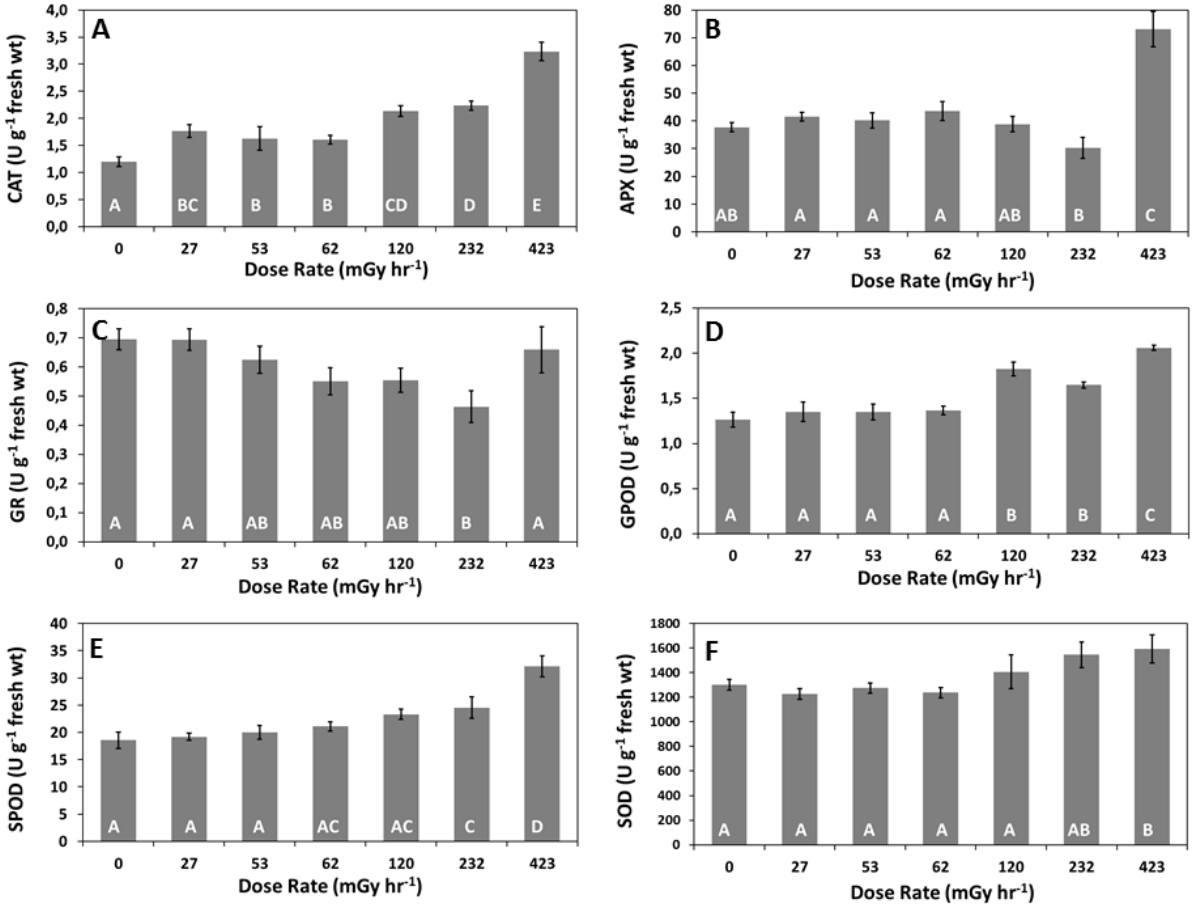

Figure 3: Enzyme activities of (A) catalase (CAT), (B) ascorbate peroxidase (APX), (C) glutathione reductase (GR), (D) guaiacol type peroxidase (GPOD), (E) syringaldizine peroxidase (SPOD) and (F) superoxide dismutase (SOD) in *L. minor* exposed to different dose rates of gamma radiation for 7 days. Each data point represents the mean ± SE of four biological replicates. Different capital letters indicate significant differences between treated plants and control plants ($p<0,05$, one-way ANOVA).

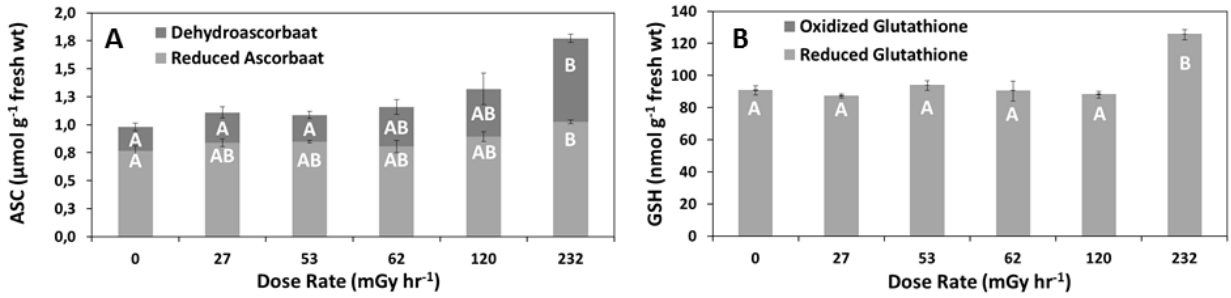

Figure 4:Metabolite concentrations of (A) Ascorbate (ASC) and (B) Glutathione (GSH) concentrations in *L. minor* exposed to different dose rates levels of gamma radation for 7 days. Concentration levels of reduced and oxidized forms of the metabolites are shown in light and dark grey bars respectively. Each data point represents the mean ± SE of four biological replicates. Different capital letters indicate significant differences between treated plants and control plants ($p<0,05$, one-way ANOVA).

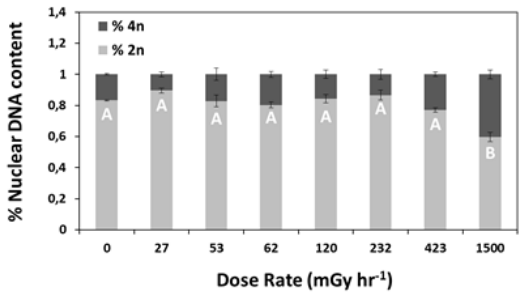

Figure 5: Relative nuclear DNA content of *L. minor* exposed to different dose rates of gamma radation for 7 days. Data points of a certain n-value (ploidy level) are given as mean percentages ± SE of four biological replicates. Different capital letters indicate significant differences between treated plants and control plants ($p<0.05$, one-way ANOVA).