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Low Doses of Gamma Irradiation Stimulate Synthesis of Bioactive Compounds with Antioxidant Activity in *Fomes fomentarius* Living Mycelium

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Abstract: Environmental changes generate free radicals and reactive oxygen species (ROS), resulting in abiotic stress in plants and fungi. Gamma ionizing radiation generates a significant amount of free radicals and ROS, thereby simulating natural environmental stressors. We used a ^{60}Co source of radiation to experimentally induce oxidative stress in living mycelium mass of the medicinal fungus *Fomes fomentarius*, in order to obtain a late response of stress tolerance by means of bioactive compounds synthesis. We measured the response at 24, 48, and 72 h after the irradiation. The highest improvement was found 24 h after exposure for antioxidant activity and for total phenolic compounds of methanolic extract, with a 1.89- and 1.64-fold increase, respectively. The total flavonoids in methanolic extract increased 1.68 times after 48 h from treatment and presented a more stable raising in the assessed time-lapse. For the three analyzed parameters, 300 Gy was the optimum absorbed dose to trigger a beneficial response, with potentially applications in pharmaceutics and nutraceutics. Gamma irradiation can be used as a biotechnological tool to direct the secondary metabolites synthesis upregulation in medicinal mushroom living mycelium.

Keywords: mycelium; antioxidant activity; gamma irradiation; *Fomes fomentarius*; mushroom; DPPH; flavonoids; polyphenols

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1. Introduction

Environmental changes generate free radicals and reactive oxygen species (ROS), resulting in abiotic stress in plants and fungi, also acting as natural regulators. This causes alterations in many metabolic pathways, especially those related to oxidative stress management [1].

Both polyphenols and flavonoids are chemical compounds, synthesized by the body, involved in antioxidant defense. They can block reactive oxygen species that lead to the onset of oxidative stress, which disorganizes the cell membrane and cell organs. Increased oxidative stress can lead to degradation of DNA molecules and oxidation of histones [2,3].

Gamma irradiation is itself considered to be a physically induced stress on living organisms or cells. Radiation treatment can be a much faster way to quantitatively improve the chemical synthesis of antioxidant compounds that may play a role in defending irradiated tissue [4,5].

Exposure to solar ultraviolet radiation and to atmospheric ozone activates both enzymatic and non-enzymatic antioxidant defense systems in response. Some flavonoids and phenolic compounds help in protecting the plants and their photosynthetic tissues from UV-B induced damages [1].

Gamma ionizing radiation generates significant amount of free radicals and ROS [6], thereby simulating natural environmental stressors.

Fomes fomentarius (tinder fungus) is a medicinal lignivorous tough polypore, producing a white-mottled rot mainly on beeches and birches. This wood-decaying macrofungus is well-known for its potential use in a wide range of biotechnological applications [7]. Its perennial woody fruiting body is shown to have cytotoxic activity against murine sarcoma S180 in vitro and to inhibit in vivo tumor growth activity of the same line cell [8]. Its ethanol extract exerts inhibition of cell growth and motility induction of apoptosis via targeting AKT in human breast cancer MDA-MB-231 cells and also decreases cell viability in six cancer cell lines; the fungus is also known to have anticancer, anti-inflammatory, and anti-diabetes effects [9,10].

According to Zhang et al., (2021) [11], the main chemical constituents of this fungus were suggested to be triterpenoids and ergosterols. The authors isolated new structures: two pentacyclic lupane-type triterpenes, 3-formyloxybetulin and 3-formyloxybetulinic acid; two rare carbon-degraded ergosterol derivatives, pyropolincisterols (A and B); along with ten known triterpenids and four erigosterols.

The most significant phenolics in *F. fomentarius* methanolic extract are benzoic acid, rutin, quercetin and protocatechuic acid; p-hydroxy benzoic acid, catechin, syringic acid, p-coumaric acid, benzoic acid, cinnamic acid, p-hydroxy benzoic acid, and vanillic acid were also found [12].

Gamma irradiation is an established sterilization method for medical devices and one which is particularly suited to plastics. A more recent application has been in the sterilization of single-use disposable components [13]. Due to DNA sensitivity, gamma irradiation was also used in plant mutation breeding, to generate crop varieties with improved treats [14,15] by selecting for useful permanent mutation in the genetic material. This latter application was however gradually abandoned in favor of targeted mutagenesis. Nowadays, gamma irradiation treatment for the improvement of various biological-active properties begins to develop as a result of its accessibility, due to the spread of industrial irradiators.

We used a ^{60}Co source of radiation to experimentally induce oxidative stress in living mycelium of *Fomes fomentarius* fungus, in order to obtain a late response of oxidative stress tolerance after treatment. We measured the response by mean of free radical scavenging capacity (RSC) on DPPH, total phenols, and total flavonoids in methanolic extract, at 24, 48, and 72 h after the irradiation exposure of living mycelium. Our aim was to find out in what range sublethal doses of gamma irradiation can be used as a biotechnological tool to elevate the level of some classes of compounds with antioxidant capacity in *Fomes fomentarius* mycelium.

2. Materials and Methods

2.1. Chemicals and Equipment

All chemicals used were purchased from Sigma—Aldrich Co. LLC St. Louis, MO, USA. Deionized water was used for the steps where it was needed. Absorbances were measured, using SpectraMax i3x Multimode Detection Platform (Molecular Devices LLC, San Jose, CA, USA). The 10 L bioreactor BIOSTAT® B (Sartorius Stedim Biotech, Göttingen, Germany) was used to obtain biomass.

2.2. Fungal Identification

The strain used in this work was isolated as pure culture dikaryotic mycelium from an active growing area of a young basidiocarp. It was harvested in a hardwood of a hilly area of Romania and kept under in vitro culture by periodic transfer on fresh culture media. The isolate was designated as FPM and was identified morphologically and confirmed by 18S ribosomal RNA gene sequencing (98% homology in NCBI database) as *Fomes fomentarius*.

2.3. Mycelial Biomass Generation

Fomes fomentarius mycelium was used as a pure strain. Stock cultures were maintained on Petri dishes on Potato Dextrose Agar (PDA), incubated at 23.5 °C.

Fresh mycelial biomass for irradiation was obtained by submerged fermentation in bioreactor in a total volume of 10 L PDB medium (Potato Dextrose Broth).

To generate the inoculum for the bioreactor culture, mycelium of *F. fomentarius* from a liquid culture was transfer to fresh PDB in Duran bottles, with Teflon membranes screw cap (pores of 0.45 µm—for sterile air passage). The experiment was performed under the following conditions: temperature of 23.5 °C, shaking speed of 100 rpm, and initial pH = 5.6 [16].

After approximately one month of incubation, the mycelium occupied the entire culture volume (400 mL). The bioreactor inoculum was prepared by triturating the biomass with sterile glass beads (Ø = 3 mm). The resulted mycelial suspension was aseptically inoculated into the bioreactor glass vessel. The experiment was performed under aeration 2 L/min, continuously.

After 7 days of growth, the bioreactor culture was ended, and the mycelium was drained through sieves with pores of 300 µm and 125 µm, then washed with deionized water to remove culture medium, and fairly drained again by settling. No vacuum pressure was applied, so the biomass was fully hydrated.

The drained biomass mycelium was divided into 4 replicative aliquots for each dose (20—500 Gy) and 8 replicative aliquots for control (0 Gy) (~ 50 g each), distributed in sterile 90 mm Petri dishes for irradiation.

2.4. Irradiation of the Mycelium of Fomes fomentarius

The irradiation was performed by using a ^{60}Co research irradiator GC-5000 (B.R.I.T.—Mumbai, India) located in IRASM Radiation Processing Department of "Horia Hulubei" National Institute of Physics and Nuclear Engineering (Romania).

The dose interval was chosen on the base of previous experiments with fungi, trying to cover from non-effective to saturating effect. Mycelial samples were acutely exposed to gamma rays at following average doses: 0 (control), 20, 50, 70, 100, 200, 300, 400, and 500 Gy, respectively, at a dose rate of 0.8 Gy/s. An alanine dosimetry system was used for dose evaluation. The reference material for the doses is water. Irradiation temperature, as measured inside the irradiation chamber, was in the range of 27–28 °C.

After irradiation, mycelial biomass aliquots were incubated at 23.5 °C for 24, 48, and 72 h, to allow metabolic recovery and response, then lyophilized.

2.5. Preparation of Samples and Obtaining the Extract

From each irradiated aliquot, three technical replicates of lyophilized mycelium were established. Each replicate of was separately ground and extracted twice in 2 mL of 80% methanol, with an incubation of 2 h, at −20 °C, each. The second extraction was preceded by sonication for 15 min. Both supernatants were combined and washed with n-hexane (*v:v*) to remove lipids and waxes.

After a final centrifugation to remove possible debris, the supernatant was evaporated to dryness and redissolved in 80% methanol. The obtained extract was stored at 4 °C until the assays were performed.

2.6. Evaluation of the Antioxidant Capacity of the Extract

2.6.1. Total Polyphenols Content (TPC)

The total content of polyphenols in the methanolic extracts of submerged cultivated mycelium after irradiation treatment was determined by using the Folin–Ciocalteu method [17]. This is based on the reduction of the Folin–Ciocalteu reagent by phenolic compounds, which will form chromogens that can be detected spectrophotometrically.

The polyphenols in *F. fomentarius* extract or gallic acid (3,4,5-trihydroxybenzoic acid) reacted with the Folin–Ciocalteu reagent, forming a blue complex which was quantified by measuring the absorbance at λ = 765 nm. Gallic acid has been used as a standard antioxidant compound for spectrophotometric determination of antioxidant activity. Its

standard curve comprised 6 dilutions: 0.05, 0.1, 0.15, 0.2, 0.25, and 0.5 mg/mL, starting from a 1 mg/mL stock solution.

The total content of polyphenols in the 20 µL sample/reference substance was determined by adding 120 µL of Folin–Ciocalteu solution was diluted 1/10, followed by 1 min of incubation at room temperature. Subsequently, 120 µL of 7.5% Na_2CO_3 solution was added, and the final mixture was incubated for 60 min, at 25 °C. Finally, O.D. was measured at 765 nm, using 80% methanol as a blank [18,19].

The total polyphenol content of the methanolic extract was calculated based on the regression equation y = 0.0057x + 0.0955 and expressed in micrograms of gallic acid equivalents per mL extract (µg GAE/mL methanolic extract) [19].

Both the samples and the reference substance were worked in triplicate, and their average absorbance value was calculated.

2.6.2. Total Flavonoids Content (TFC)

The flavonoid content of methanolic extracts obtained from mycelium of *F. fomentarius* was quantified according to the colorimetric method of aluminum chloride, described by Lamaison and Carnet (1990) [20]. To achieve the reaction, 2.5% $AlCl_3$ solution was added over the sample/standard quercetin solution (in a 1:1 volumetric ratio), and the mixture was incubated at room temperature for 20 min. The standard curve was made of quercetin and comprised 5 dilutions (0.01, 0.02, 0.03, 0.04, and 0.05 mg/mL), starting from a stock solution of 1 mg/mL in 80% methanol. The absorbance was read at 420 nm, and the flavonoid content was calculated based on the regression equation y = 31.538x − 0.5262 and expressed in µg of quercetin equivalents (QE) per mL of extract [21].

2.6.3. The DPPH Method

The evaluation of the antioxidant properties of the methanolic extract obtained from the submerged mycelium of *F. fomentarius* was determined by using the radical 2,2-diphenyl-1-picrilhydrazyl (DPPH), according to the method of Burits [22].

The sample was reacted with the 0.25 mM DPPH solution in 80% methanol, in a ratio of 1/10 = *v/v*. The optical density was measured at 523 nm after incubation of the plates, in the dark, for 40 min, at room temperature.

DPPH reduction occurs when it reacts with an antioxidant compound that can donate hydrogen. Thus, the color varies with the reduction of DPPH (from purple to pale yellow), and the color intensity decreases depending on the concentration of antioxidant in the sample.

The free radical scavenging activity of DPPH (expressed as a percentage) was calculated by using the following formula [23]:

$$\% \text{ inhibition} = [(A_{blank} - A_{sample})/A_{blank}] \times 100$$

where A_{blank} = absorbance blank, and A_{sample} = absorbance sample.

2.7. Statistical Analysis

All extracts were performed in three technical replicates for each dose and each response period (time-lapse after irradiation), and each replicate was read three times (n = 120). Mean values were used to calculate antioxidant activity and standard deviation (SD) values for each experimental group. Comparisons between groups (control and mycelium subjected to gamma irradiation) were evaluated by one-way ANOVA. A value of $p < 0.05$ was considered statistically significant. The graphics were plotted by using OriginLab 9.0.

3. Results

3.1. Statistics

Both gallic acid and quercetin calibration curve rendered a very good correlation coefficient (R^2 = 0.9986 and R^2 = 0.9997, respectively).

A vast majority of results in the range of 200 to 300 Gy differ significantly from the non-irradiated control; statistical difference between response times can also be noticed, as per Table 1.

Table 1. A one-way ANOVA.

Assay	Compared Variables (Irradiation Doses)	24 h	48 h	72 h
		Statistical Significance of the Difference (*p*-Value)		
DPPH	0 Gy/200 Gy	0.0001	0.0014	0.0532
	0 Gy/300 Gy	0.0000	0.0000	0.0003
	0 Gy/400 Gy	0.0000	0.0002	0.0007
	0 Gy/500 Gy	0.0001	0.0003	0.0027
TPC	0 Gy/200 Gy	0.0000	0.0036	0.7250
	0 Gy/300 Gy	0.0000	0.0001	0.5286
	0 Gy/400 Gy	0.0000	0.0086	0.0382
	0 Gy/500 Gy	0.0000	0.0073	0.0006
TFC	0 Gy/200 Gy	0.0200	0.0006	0.6781
	0 Gy/300 Gy	0.0086	0.0254	0.0000
	0 Gy/400 Gy	0.0010	0.0433	0.0204
	0 Gy/500 Gy	0.1100	0.0879	0.0000

Although differences in results between treated samples at very low doses (20–100 Gy) and untreated control turned partially statistically insignificant (Table 2), a general trend for all tested parameters can be observed, namely that doses under 100 Gy proved inefficient in upregulating synthesis of active metabolites.

Table 2. A one-way ANOVA.

Assay	Compared Variables (Irradiation Doses)	24 h	48 h	72 h
		Statistical Significance of the Difference (*p*-Value)		
DPPH	0 Gy/20 Gy	0.0234	0.0012	0.0186
	0 Gy/50 Gy	0.1220	0.0003	0.0719
	0 Gy/70 Gy	0.6468	0.0001	0.0045
	0 Gy/100 Gy	0.0720	0.0000	0.0004
TPC	0 Gy/20 Gy	0.0250	0.0929	0.8858
	0 Gy/50 Gy	0.1468	0.0183	0.2233
	0 Gy/70 Gy	0.0002	0.6792	0.1678
	0 Gy/100 Gy	0.0006	0.0001	0.0002
TFC	0 Gy/20 Gy	0.2837	0.1741	0.5092
	0 Gy/50 Gy	0.0447	0.0162	0.0146
	0 Gy/70 Gy	0.0304	0.0026	0.4435
	0 Gy/100 Gy	0.0007	0.0029	0.0012

3.2. DPPH Method

Our work shows that a delay after the irradiation treatment, prior to extraction, increases the extract's capacity of scavenging the DPPH free radical (Figure 1) from 18.5 ± 1.88% (untreated control) to a range of 28.83 ± 1.4–34.99 ± 1.14% (irradiated) af-

ter 24 h, from 29.8 ± 1.45% (untreated control) to a range of 38.06 ± 1.6–45.48 ± 2.45% (irradiated) after 48 h, and from 51.07 ± 2.53% (untreated control) to a range of 54.90 ± 1.94–60.19 ± 0.6% (irradiated) after 72 h. Overall, this represents a maximum mean enhancement of 1.89 times of scavenging capacity, recorded after 24 h, at 300 Gy (Figure 1). The three tested response times shown the maximum increase at 300 Gy. Very low doses, in the range of 20 to 100 Gy, had negative or non-significant effect on the antioxidant activity (Figure 1).

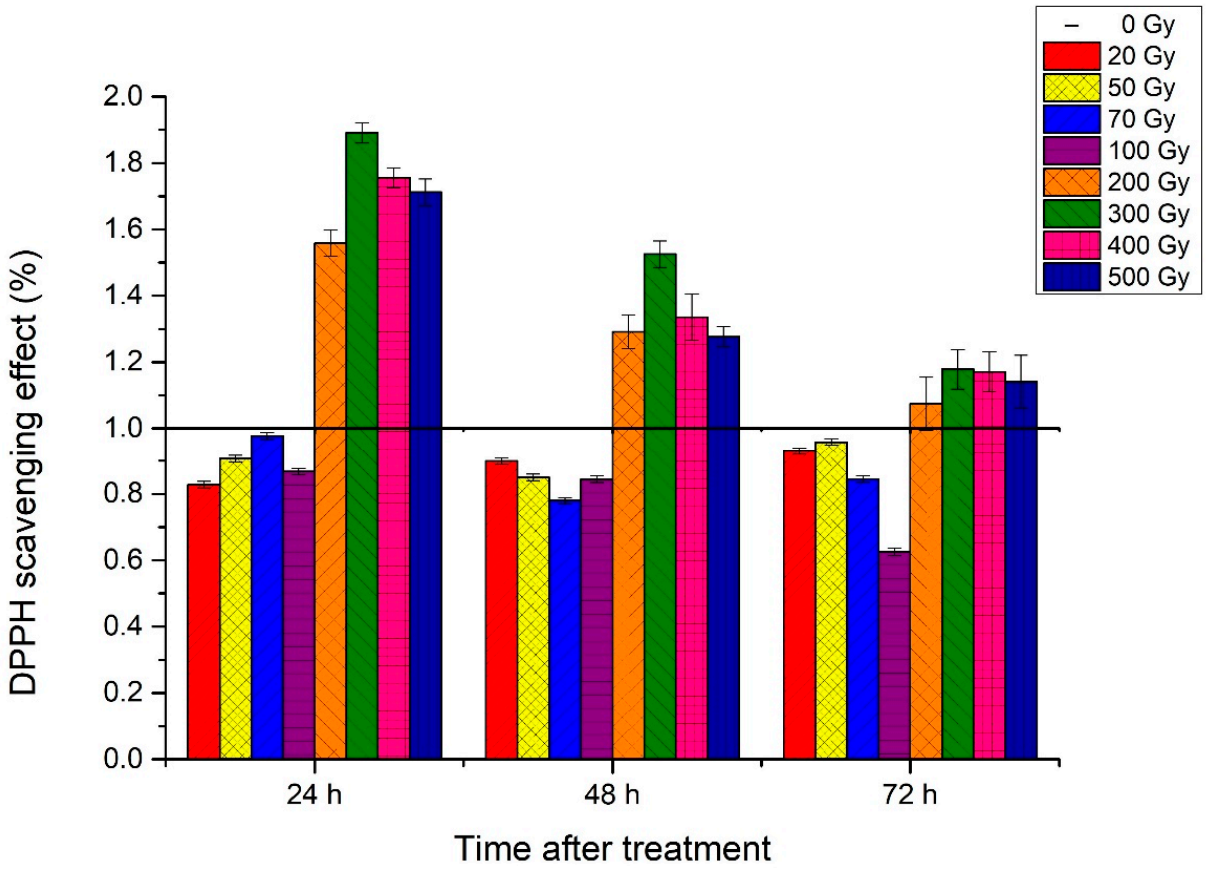

Figure 1. The effect of gamma irradiation and response-time on the antioxidant capacity in methanolic extract of *Fomes fomentarius* mycelium. Results are expressed as average irradiated/untreated control ratio (n = 120 per time). Bars are one standard deviation (SD).

3.3. Total Polyphenols Content (TPC)

In the range of 200 to 500 Gy, the total polyphenols content of methanolic extract increases from 241.14 ± 7.25 (untreated control) to a range of 312.95 ± 9.98–396.14 ± 20.83 µg GAE/mL (irradiated) after 24 h, from 375.64 ± 23.42 (untreated control) to a range of 431.43 ± 15.48–500.22 ± 14.48 µg GAE/mL (irradiated) after 48 h, and from 551.18 ± 26.29 (untreated control) to 560.64 ± 10.42 µg GAE/mL (in samples irradiated at 300 Gy) after 72 h. Overall, this represent a maximum mean enhancement of 1.64 times of total polyphenolic content, recorded after 24 h, at 300 Gy (Figure 2). Again, the three tested response times shown the maximum increase at 300 Gy. Very low doses (20–100 Gy) produced irrelevant variations in total polyphenols (Figure 2).

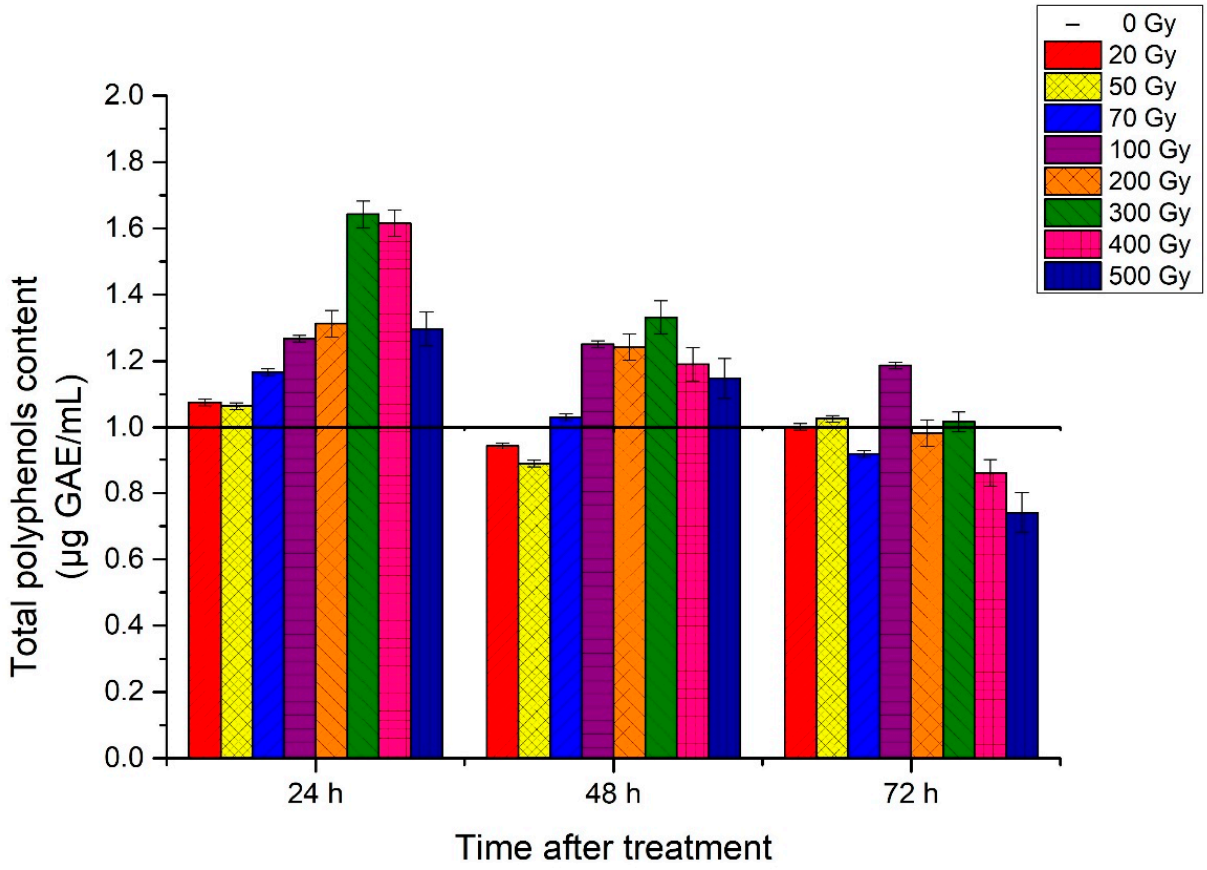

Figure 2. The effect of gamma irradiation and response-time after treatment on the total content of polyphenols in methanolic extract of *Fomes fomentarius* mycelium. Results are expressed in gallic acid equivalent as average irradiated/untreated control ratio (n = 120 per time). Bars are one standard deviation (SD).

3.4. Total Flavonoids Content (TFC)

For the total flavonoids content of methanolic extract, we encountered improvement in all doses. The significant differences over control were obtained in the range of 200 to 500 Gy, as follows: from 12.98 ± 0.72 (untreated control) to a range of 15.09 ± 1.22–21.63 ± 1.99 µg quercetin/mL (irradiated) after 24 h, from 14.84 ± 0.74 (untreated control) to a range of 18.6 ± 0.75–25.06 ± 0.67 µg quercetin/mL (irradiated) after 48 h, and from 10.44 ± 0.75 (untreated control) to a range of 11.24 ± 0.23–16.72 ± 1.79 µg quercetin/mL (irradiated) after 72 h. Overall, this represent a mean maximum enhancement of 1.68 times of total flavonoids, recorded after 48 h, at 300 Gy (Figure 3). As with previous measurements, the three tested response times shown the maximum increase at 300 Gy. Furthermore, a significant increase in flavonoids content was also generated by 100 Gy: 1.61 irradiated/control ratio after 24 h, 1.56 after 48 h, and 1.36 after 72 h. Irrelevant upregulations were recorded at doses under 100 Gy.

3.5. Response Time after Irradiation

Since the maximum yield of Total Polyphenols Content and DPPH scavenging effect of methanolic extract was obtained after 24 h, it is plausible that a shorter delay may reveal an even greater increase of these parameters, provided that optimal living conditions are ensured. For those parameters, the maximum response slowly quenches in time, with a difference of 1.53 fold after 48 h and 1.18 fold after 72 h, above the untreated control for DPPH scavenging, and a difference of 1.33 fold above the untreated control after 48 h and no difference after 72 h for total polyphenols.

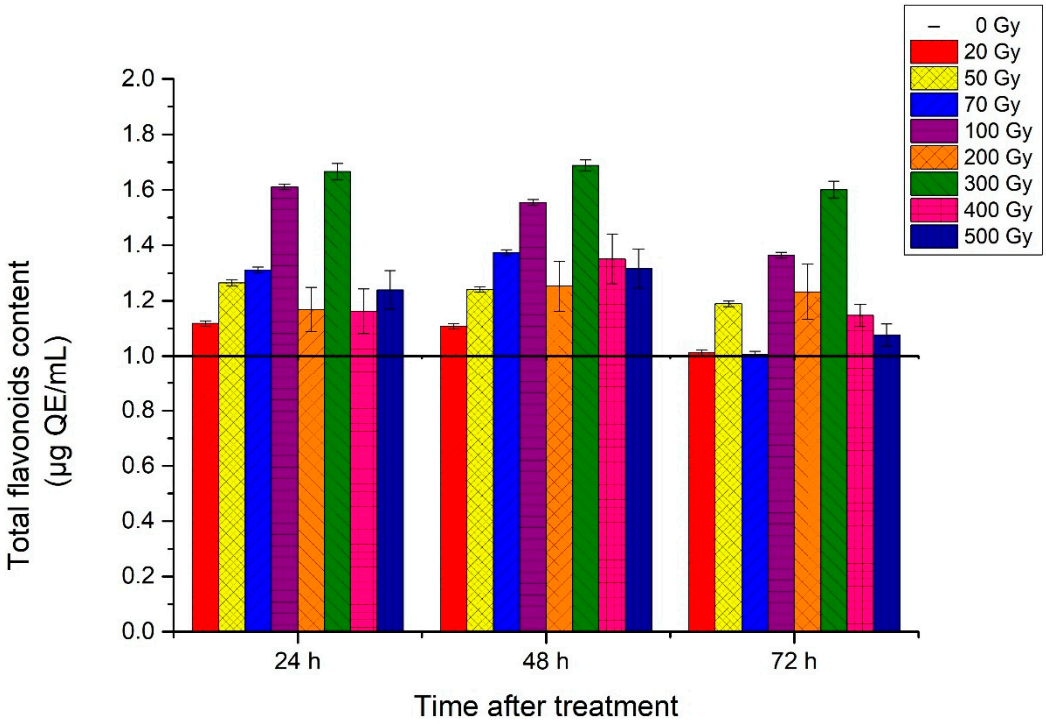

Figure 3. The effect of gamma irradiation and response-time after treatment on the total flavonoids content in methanolic extract of *Fomes fomentarius* mycelium. Results are expressed in quercetin equivalent as average irradiated/untreated control ratio (n = 120 per time). Bars are one standard deviation (SD).

Our results also show that the antioxidant capacity (DPPH free radical scavenging) and total polyphenols level increase over time, regardless of the irradiation dose, due to their extracellular nature. Instead of being diluted by the nutritive medium while in culture, secondary metabolites concentrate as a jelly matrix in the mycelial mass, outside the hyphae, partially consisting of exopolysaccharide (EPS), which were shown to present free radical scavenging activity [24]. Flavonoids instead, being accumulated in-cell, are consumed and in constant decline over time, regardless of the irradiation dose. The difference between doses however, maintains in flavonoids, in contrast to the total polyphenols and antioxidant activity by DPPH assay, which tends to fade after 72 h.

Response in terms of flavonoids synthesis showed more stable over time at all doses, with very small variation between maxima (namely increase of 1.66-fold after 24 h, 1.68- fold after 48 h, and 1.60-fold after 72 h, respectively, at 300 Gy). Flavonoids have great potential to counter the detrimental effects of the massive generation of reactive oxygen species (ROS). The notion that large alterations in the cell redox state trigger the biosynthesis of flavonoids [25,26], led Agati et al., (2020) [27] to hypothesize that flavonoids may complement the functions of primary antioxidants when their ROS detoxifying capacity declines in plants suffering from severe photo-oxidative stress. Barry Halliwell, who provided a series of definitions of "antioxidants", has always been critical of the effective ability of flavonoids to "delay, prevent or remove oxidative damage to a target molecule", a widely accepted definition of antioxidant given in the paper written by Gutteridge and Halliwell [27,28].

A general growth trend of all parameters with time was noticed, which was to be expected due to concentration of secreted secondary metabolites outside cells.

4. Discussion

Similar approaches of irradiating fresh living fungi or plants, in order to enhance their nutritional value, showed similar results, depending on the experimental design and the assessed compounds. Working with *Cordyceps militaris* medicinal fungus and using UV-B as source of radiation, Huang et al. [29] showed that the flavonoid content increased by

30.6 to 56% in living mycelium (depending on the preparation method) and by 10.4% in living fruiting bodies. UV-B irradiation also considerably increased total phenol content in mycelia, but had insignificant effect on mushrooms. However, we must notice that time after irradiation, up to the freezing step, is not mentioned and, according to our estimation, the absorbed energy (in Joules) was ~11 times higher in UV-B irradiated *Cordyceps* than in our experiment (gamma irradiated *Fomes fomentarius* mycelium).

Taheri et al. (2014) [30] found that acute gamma irradiation enriches the content of some polyphenols and flavonoids in *Curcuma alismatifolia* leaves of plants whose rhizomes have been previously irradiated. All analyzed polyphenols, namely salicylic acid, caffeic acid, catechin, epicatechin, cinnamic acid, ellagic acid, resorcinol and flavonoids, namely rutin, naringin, apigenin, quercetin, myricetin, showed an increase in a range similar to our work. Their experiment was performed at doses of about an order of magnitude smaller than ours (tens of grays), since plants are less radioresistant than fungi and their highest dose (20 Gy) presented the highest increase in both polyphenols and flavonoids.

Liu et al. [31] also found that irradiation with UV-B light after harvest enhanced the amount of flavonoids, phenols and total phenols in tomato fruits, while Du et al., [32] reported that low-dose UV-B light treatment to freshly cut carrot products could increase the total soluble phenolic content and enhance antioxidant activity. The penetration power of the two radiation sources is, however, different: while ultraviolet radiation acts only on the surface, gamma photons are highly penetrating, making results only partially comparable.

Köhler et al. [33] showed that the enzymatic (superoxide dismutase, SOD and total peroxidases, POD) and non-enzymatic antioxidant activity (total phenolic) increased significantly in *Deschampsia antarctica* plants in hydroponic system, in response to UV-B treatment. A peak in total phenolic content of *D. antarctica* was found at 3 h of exposure, which related to percentage of consumed DPPH and FRAP values, suggesting that these phenolics function as soluble antioxidant metabolites. Various polyphenols have antioxidant and anti-inflammatory properties that could have preventive and/or therapeutic effects for cardiovascular disease, neurodegenerative disorders, cancer, and obesity [34].

Submerged fermentation is a method at hand for producing fungal biomass of rare and/or slow growing species. The process is easily controllable, regardless of the season, and the total and specific compound yield are predictable. Mycelia and/or fermentation broth could be harvest in couple of days to few weeks, depending on the desired metabolite. Freeze-drying of live mycelium is a good method to both conserve the properties of compounds and to catch the differences between treatments and response time, since metabolic activity is completely interrupted.

Our experimental approach reveals gamma irradiation as a novel biotechnological tool to obtain medicinal mycelium with improved antioxidant properties. Such a natural resource is highly desirable for pharmaceutical, as well as for nutraceutical products. Due to very small doses that are necessary, industrial gamma irradiation becomes a cost-effective large-mass-applicable instrument to generate added value to raw biological material for extracts or new dietary supplements and functional foods. By density and water content, submerged cultivated mycelium (after draining) is very similar to fresh food. Doses are also in the same range, so the cost of mycelium irradiation for stimulation of bioactive compound synthesis is similar to fresh food irradiation, in industrial or semi-industrial plants; food irradiation for decontamination and shelf-life extension is a common practice, currently costing few cents per kilogram. Other oxidative stressors like exposure to ozone or hydrogen peroxide (H_2O_2) are a chemical approach with a dose-effect ratio that needs to be investigated on a case-by-case basis. Cellular interaction with heavy metal ions, such as iron, copper, cadmium, mercury, nickel, lead, and arsenic, also generates free radicals and oxidative stress, but fungi are particularly eager to absorb heavy metals potentially causing intoxications.

Stimulating fresh living mycelium with gamma irradiation is a completely different approach from irradiating either dry mycelium or even final extract of it. Apart from significantly higher doses, in the range of kGy to tens of kGy, in the latter strategy, gamma

irradiation acts by different mechanism, probably by degrading or decomposing some compounds into antioxidant components [35]. In fact, Adamo et al. [36] proposed that the destructive processes of oxidation and γ-irradiation were capable of breaking the chemical bonds of polyphenols, thereby releasing soluble phenols of low molecular weight. This may change the content and composition of antioxidant components, thereby affecting the antioxidant properties. Basically, it is not a real enrichment, since no new active biosynthesis is made, but rather an improved image of its bioactivity. In contrast, applying low oxidative stress triggers a cellular response, implying conversion of different stored precursors into metabolites with radioprotective and scavenging abilities on free radicals, meant to counteract and prevent future damages. In a certain window, this response could be used in the benefit of human welfare.

5. Conclusions

So far, our results point out that 300 Gy is the optimum gamma irradiation dose for stimulation of antioxidant response in living mycelium for all the investigated parameters (TPC, TFC, and DPPH). If an interval is to be considered for the delivered dose, to cover for inherited errors, then 400 Gy is the end of the interval. The dose may appear surprisingly high for stimulation purposes, but we should take into account that fungi, especially melanized ones, are highly radioresistant and resilient species. Anyway, for stimulation approach, irradiation dose must be chosen in the radiation resistance window of the species, in order for cellular damages to be recoverable and to allow for proper response; this dose may differ from one species to another and must be confirmed experimentally.

Based on our results, a gamma photons exposure at 300 Gy, followed by a time response, could markedly enrich the living mycelium of the medicinal fungus *Fomes fomentarius* in total polyphenols and flavonoids and could potentiate its antioxidant activity. Doses below 100 Gy proved invariably inefficient in generating any relevant effect.

The response time after treatment depends on the targeted antioxidant compound, provided that the mycelium is in good state and optimum conditions (temperature and air access) are offered.

To our knowledge, this is the first record of the usage of gamma irradiation, at small sublethal doses, as a biotechnological tool to increase the antioxidant capacity of *Fomes fomentarius* living mycelium, as proven by methanolic extract.

Author Contributions: Conceptualization methodology and formal analysis, M.E.; statistics, software, and graphs, C.F.P.; strain isolation and identification, M.E.; extractions, assays, and data acquisition, C.F.P.; irradiation treatments, D.C.N.; extraction protocol optimization, A.-M.P.; writing—original draft preparation, C.F.P.; writing—review and editing, M.E.; project administration, M.E. All authors have read and agreed to the published version of the manuscript.

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