



The use of gamma irradiation to stimulate bioactive compound synthesis in *Inonotus obliquus* submerged cultures

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Abstract. *Inonotus obliquus* is a parasite on the birch and other trees and is also a well-known medicinal mushroom. Its sterile conk is highly sought for its bioactive compounds such as phenols, polysaccharides, triterpenoids, and steroids. It was traditionally used to treat various gastrointestinal diseases, viral and parasitic infections, to counteract the progression of cancers, and to stimulate the immune system. We used acute gamma irradiation, followed by short-term submerged cultivation, as an oxidative stress inducer to enhance the synthesis of mycelial metabolites. The 300 Gy and 400 Gy doses showed the best results across the whole experimental design. Each assayed criterion had a different corresponding optimal stimulation dose. In one experiment, sublethal doses of irradiation triggered the dry weight of the cultured mycelium to increase by 19.764%. The free radical scavenging potential of the mycelium extracts increased by 79.83%. The total phenolic content of mycelium extracts and culture broth increased by 55.7% and 62.987%, respectively. The total flavonoid and sinapinic acid content of the broth increased by 934.678% and 590.395%, respectively. As such, gamma irradiation pre-treatment of the mycelial inoculum proved an interesting, economically and environmentally effective tool for stimulating secondary metabolite synthesis in submerged mycelium cultures.

Keywords: Gamma irradiation • Chaga • Medicinal fungi • Metabolite synthesis • Bioactive compounds

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Introduction

The parasitic fungus *Inonotus obliquus*, also known as the Chaga mushroom (*Hymenochaetaceae*), grows on the trunk of birch and other trees in the circumboreal region of the northern hemisphere [1], but does so very slowly, making it difficult to rely on the natural lifecycle of this mushroom for obtaining bioactive metabolites for industrial applications [2]. It produces various types of metabolites such as phenols, polysaccharides, triterpenoids, and steroids [3]. The phenolic compounds found in *I. obliquus* show great potential for the scavenging of free radicals [4]. Past research involving submerged cultures of this species has focused on the production of biomass [5] and the accumulation of phenolic compounds [6]. The great majority of research involving gamma radiation and mushrooms employs γ rays as a conditioning agent with harvested mushrooms or extracts, aiming to increase nutrient or antioxidant levels through the breakdown of existing compounds [7, 8]. In this study, we used acute gamma irradiation of submerged *I. obliquus* mycelium, followed by cultivation, as a tool to stimulate production of biologically active metabolites, in response to the oxidative stressor represented by radiation exposure, via a hormetic process. We monitored changes in

mycelial biomass and antioxidant levels, as well as the amount and class of compounds with antioxidant properties.

Methods

Mycelium cultivation and irradiation

I. obliquus (Ach. ex Pers.) Pilát (CBS 314.39) mycelium was purchased from Westerdijk Fungal Biodiversity Institute (Utrecht, The Netherlands) and grown on potato dextrose agar for seven days at 23.5°C. Subsequently, 1 cm × 1 cm agar sectors were each transferred to potato dextrose broth (PDB) seed culture medium. The mycelium was cultured at the same temperature, in 500 mL bottles with polytetrafluoroethylene (PTFE) membrane screw caps, agitated at 100 rpm for 28 days, until it occupied the entire volume of the culture vessels. In order to ensure identical inoculum for all replicates, five such fully grown colonies were brought together in a sterile 3000 mL Erlenmeyer flask and triturated; and 400 mL of the homogenized mycelium was distributed back into the culture vessels.

The application of the oxidative stress induced by gamma radiation on the mycelium of *I. obliquus*, in a liquid medium, was performed separately on each culture vessel. Sublethal doses of 0, 100, 200, 300, and 400 Gy were given to the triturated mycelium, according to the experimental design.

After the cultures were irradiated, each was used to seed five replicates of new PDB liquid cultures by transferring 80 mL of homogenized mycelium (a 20% v/v inoculum) to a total volume of 400 mL of new culture. Thus, the irradiation experiment had five doses with five replicates each, which were cultured for 16 days after being irradiated, under the same temperature and agitation parameters as before.

Gamma radiation technology and dosimetry

Irradiations were carried out using the Co-60 research irradiator GC-5000 (B.R.I.T., India), within the IRASM Radiation Processing Center, part of “Horia Hulubei” National Institute of Physics and Nuclear Engineering, Romania.

We used an alanine-EPR dosimetry system to evaluate doses. The irradiations were performed at an average dose rate of 0.8 Gy/s with an associated relative uncertainty of 3%. The dose uniformity ratio, defined as the ratio of the maximum to the minimum dose in a set of samples, was about 1.6. All doses are expressed as absorbed dose in water.

Mycelial biomass and fermented broth harvesting

16-day old cultures were harvested by separating the mycelial biomass from the fermented broth through a 500 nm stainless steel sieve, followed by further filtration of the broth through a 180 mm Nylon Net (Merck Millipore), under vacuum. The mycelia were

then washed three times with deionized water and frozen at -50°C before being freeze dried. Mycelial biomass was estimated by dry weight.

Methanolic extraction of the dry mycelium

100 mg of each irradiated mycelium was milled to a powder, distributed to 2 mL microtubes, and extracted two times in cold methanol. The dried biomass was kept in 2 mL of 80% cold methanol for 24 h at -50°C, and then the tubes were agitated on a horizontal shaker at 1300 rpm for 30 min and centrifuged for 10 min at 14 000 rpm. The two supernatants were saved and reunited in the end.

To remove lipids, each extract was washed three times with an equal volume of hexane, discarding the organic phase between washes. After removing the last volume of hexane, the extracts were evaporated at 40°C and then redissolved in 1 mL of 80% methanol on a horizontal shaker at 1300 rpm. Ultimately, the extracts were then centrifuged for 10 min at 14 000 rpm to remove any undissolved particles.

Colorimetric assays

Negative controls were prepared using distilled water or 80% methanol, depending on the sample solvent. Samples were reacted three times with the colorimetric reagents and quantified twice. Results were calculated as an average of these technical replicates.

DPPH free radical scavenging assay

The free radical scavenging potential of the mycelium extracts was determined using a modified DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) colorimetric method [9]. A 0.125 mM DPPH (TCI Chemicals) solution was prepared using a 4:1 (v/v) methanol-PBS mixture, of which 295 mL were reacted with 5 mL of each extract. The reaction mixture was kept at room temperature, in darkness, for 30 min. The absorbance of the reaction mixture was recorded at 517 nm using a SpectraMax i3x (Molecular Devices) plate reader.

The calibration curve (0–500 mg/mL) was plotted using ascorbic acid dissolved in 80% methanol as a standard. The free radical scavenging potential was expressed as milligram of ascorbic acid equivalent per milliliter.

Total phenolic quantification assay

Both extra- and intra-cellular total phenolic compounds (EPC and IPC) were quantified from culture broth and mycelium extract, respectively.

The total phenolic content was determined by colorimetric methods [10] with Folin–Ciocalteu reagent (Sigma-Aldrich). A 10-fold dilution of this reagent and a 7.5% (w/v) Na₂CO₃ solution were prepared using deionized water. A 200 mL sample was mixed with 1 mL of the diluted Folin–Ciocalteu reagent, followed by 1 mL of sodium carbonate solution. The

reaction mixture was kept at room temperature, in darkness, for 30 min. The absorbance of the reaction mixture was recorded at 765 nm using a SpectraMax i3x plate reader.

The calibration curve (0–200 mg/mL) was plotted using gallic acid as a standard. The total phenols were expressed as microgram of gallic acid equivalent per milliliter.

Total flavonoid quantification assay

The total flavonoid content of the whole crude fermentation (culture) broth was determined using a modified aluminium chloride colorimetric method [11]. A 2.5% (w/v) AlCl₃ solution was prepared using 50% (v/v) ethanol, of which 1 mL was mixed with an equal volume of a fermented broth sample. The reaction mixture was kept at room temperature, in darkness, for 30 min. The absorbance of the reaction mixture was recorded at 420 nm using a SpectraMax i3x plate reader.

The calibration curve (0–50 mg/mL) was plotted using quercetin as a standard. The total flavonoids were expressed as microgram of quercetin equivalent per milliliter.

Capillary zonal electrophoresis analysis of polyphenols

Sample preparation

The fermentation broths were subjected to a solid phase extraction (SPE) procedure to obtain concentrated extracts in polyphenols. Chromabond cartridges (Macherey–Nagel, 500 mg) were used. The stationary phase of the cartridges was activated by successive washes with methanol and water, after which 30 mL of each sample was added. The elution of the polyphenols from the cartridges was performed with 10 mL of methanol. The extracted samples were evaporated to dryness in a turbovap (Eppendorf) at 30°C, and the residue thus obtained was dissolved in 100 mL methanol, centrifuged, and injected into the capillary electrophoresis apparatus. The results are given as the average of three injections.

Capillary zonal electrophoresis

Polyphenols separation was performed on an Agilent CE system with a diode array detector. A standard bare fused-silica capillary (I.D. 50 μm, effective length of 72 cm) was used for separation. Before each sample migration, the capillary was washed with 0.1 M NaOH, ultrapure water, and migration buffer. The sample was injected in hydrodynamic mode (3500 Pa, 12 s) and the capillary was maintained at 30°C. The migration buffer consisted of a 50 mM sodium tetraborate and 0.9 mM sodium dodecyl sulfate (SDS) solution, with the pH adjusted to 9.15 using 1 M HCl. The applied voltage was 30 kV and the detection was performed in the 200–360 nm range [12]. The quantification of the compounds was performed by measurements at a 280 nm wavelength. The data have been processed using the ChemStation (Agilent) application for data analysis.

Results and discussion

Analysis of the effect of gamma irradiation on mycelial biomass

The gamma irradiation of mycelial cultures and subsequent biomass analysis were performed in duplicate, each experiment containing 25 shake flask cultures (five doses with five replicates each). Results from both experiments are shown separately.

For the first experimental run, after irradiation and 16 days of shake flask culture, the dry mycelial biomass presented an increase of 4.572–19.764% in the irradiated experimental variants, depending on the dose, compared to the non-irradiated controls.

The statistical significance between the irradiated groups in terms of dry mycelium weight was evaluated by analysis of variance (Table 1). The result demonstrates that the differences are statistically highly significant ($F = 12.520$, and $P < 0.001$).

To identify the groups between which these differences are found, and to ascertain the degree of significance for each comparison, the Tukey–Kramer *post-hoc* test was performed. The result showed very significant ($P < 0.010$) differences between the

Table 1. The one-way ANOVA of dry mycelium weight after irradiation and culture of the first experimental run

Groups	Count	Sum	Average	Variance	Std. deviation	Std. error
0 Gy	5	6.78	1.356	0.00468	0.068411	0.030594
100 Gy	5	7.09	1.418	0.00342	0.058481	0.026153
200 Gy	5	7.73	1.546	0.00753	0.086776	0.038807
300 Gy	5	8.07	1.614	0.00863	0.092898	0.041545
400 Gy	5	8.12	1.624	0.00433	0.065803	0.029428
ANOVA single factor ($\alpha = 0.01$)						
Source of variation	SS	df	MS	F	P-value	F crit.
Between groups	0.286376	4	0.071594	12.52081147	0.000029182	4.430690161
Within groups	0.114360	20	0.005718			
Total	0.400736	24				

SS, sum of squares; df, degrees of freedom; MS, mean square.

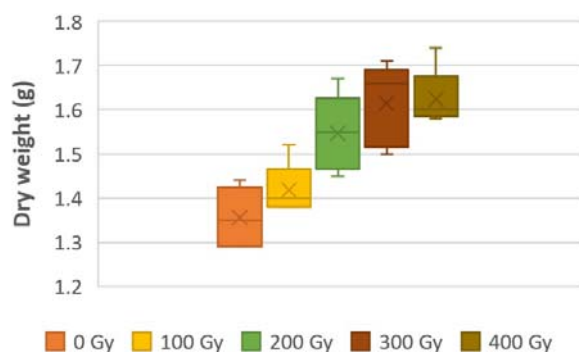


Fig. 1. The radiation dose-dependent distribution of dry mycelium weight per 400 mL of culture, in the first experimental run.

following groups: 0 Gy vs. 200, 300, and 400 Gy; 100 Gy vs. 300 Gy and 400 Gy.

The distribution of dry mycelium weight within and between irradiated groups is represented in Fig. 1.

For the second experimental run, after irradiation and 16 days of shake flask culture, the dry mycelial biomass presented an increase of 8.586–16.630% in the irradiated experimental variants, depending on the dose, compared to the non-irradiated controls.

The statistical significance between the irradiated groups in terms of dry mycelium weight was evaluated by analysis of variance (Table 2). The result demonstrates that the differences are statistically highly significant ($F = 13.286$ and $P < 0.001$).

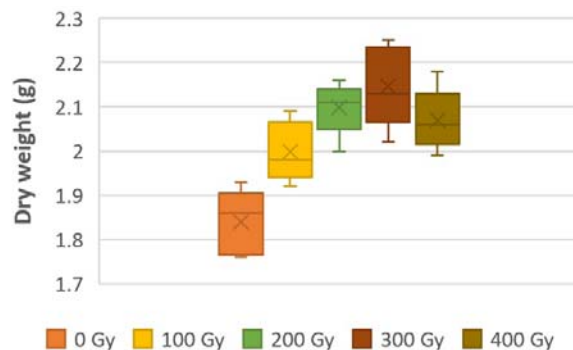


Fig. 2. The radiation dose-dependent distribution of dry mycelium weight per 400 mL of culture, in the second experimental run.

To identify the groups between which these differences are found, and to ascertain the degree of significance for each comparison, the Tukey–Kramer *post-hoc* test was performed. The result showed very significant ($P < 0.010$) differences between the following groups: 0 Gy vs. 200, 300, and 400 Gy. Significant ($P < 0.050$) differences were also found between the 0 Gy vs. 100 Gy groups; and 100 Gy vs. 300 Gy groups.

The distribution of dry mycelium weight within and between irradiated groups is represented in Fig. 2.

Table 2. The one-way ANOVA of dry mycelium weight after irradiation and culture of the second experimental run

Groups	Count	Sum	Average	Variance	Std. deviation	Std. error
0 Gy	5	9.20	1.840	0.00535	0.073144	0.032710
100 Gy	5	9.99	1.998	0.00452	0.067231	0.030066
200 Gy	5	10.49	2.098	0.00352	0.059330	0.026532
300 Gy	5	10.73	2.146	0.00843	0.091815	0.041060
400 Gy	5	10.35	2.070	0.00490	0.07	0.031304

ANOVA single factor ($\alpha = 0.01$)

Source of variation	SS	df	MS	F	P-value	F crit.
Between groups	0.284016	4	0.071004	13.28667665	0.000019303	2.866081402
Within groups	0.106880	20	0.005344	0.10688		
Total	0.390896	24				

SS, sum of squares; df, degrees of freedom; MS, mean square.

Table 3. The one-way ANOVA of free radical scavenging potential of the mycelium extracts

Groups	Count	Sum	Average	Variance	Std. deviation	Std. error
0 Gy	5	1.3562	0.2712	0.0019	0.044382	0.019848
100 Gy	4	1.6489	0.4122	0.0011	0.034140	0.017070
200 Gy	5	2.1722	0.4344	0.0008	0.029030	0.012983
300 Gy	5	2.4386	0.4877	0.0025	0.050210	0.022455
400 Gy	5	2.3953	0.4790	0.0014	0.038588	0.017257

ANOVA single factor ($\alpha = 0.01$)

Source of variation	SS	df	MS	F	P-value	F crit.
Between groups	0.152096309	4	0.038024077	23.466281	0.0000003965	4.500257699
Within groups	0.030787046	19	0.001620371			
Total	0.182883355	23				

SS, sum of squares; df, degrees of freedom; MS, mean square.

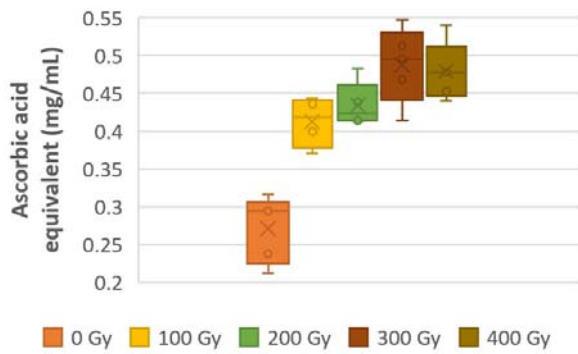


Fig. 3. The radiation dose-dependent distribution of free radical scavenging potential of the mycelium extracts.

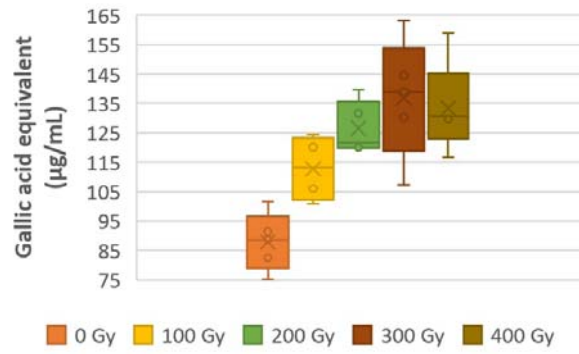


Fig. 4. The radiation dose-dependent distribution of total phenolic content of the mycelium extracts.

Determination of the free radical scavenging activity

The DPPH free radical scavenging activity of the irradiated mycelium extracts showed a 51.99–79.83% increase, depending on the dose, in comparison with non-irradiated control.

The statistical significance between the irradiated groups in terms of free radical scavenging potential of the mycelium extracts was evaluated by analysis of variance (Table 3). The result demonstrates that the differences are statistically highly significant ($F = 23.466$ and $P < 0.001$).

To identify the groups between which these differences are found, and to ascertain the degree of significance for each comparison, the Tukey–Kramer *post-hoc* test was performed. The result showed very significant ($P < 0.010$) differences between the following groups: 0 Gy vs. 100, 200, 300, and 400 Gy.

The distribution of free radical scavenging potential of the mycelium extracts within and between irradiated groups is represented in Fig. 3.

Determination of the total phenolic content

The total phenolic content of the mycelium methanolic extract increased by 28.4–55.7%, depending on the dose, in comparison with non-irradiated controls.

The statistical significance between the irradiated groups in terms of total phenolic content of the mycelium extracts was evaluated by analysis of variance (Table 4). The result demonstrates that the

differences are statistically highly significant ($F = 10.167$ and $P < 0.001$).

To identify the groups between which these differences are found, and to ascertain the degree of significance for each comparison, the Tukey–Kramer *post-hoc* test was performed. The result showed very significant ($P < 0.010$) differences between the following groups: 0 Gy vs. 200, 300, and 400 Gy.

The total phenolic content of the mycelium extracts within and between irradiated groups is represented in Fig. 4.

Furthermore, the extra-cellular total phenolic content was also estimated by the same method for the crude fermentation broth. As with the mycelium extracts, the statistical significance between the irradiated groups was evaluated by analysis of variance (Table 5). The result demonstrates that the differences are statistically highly significant ($F = 15.102$ and $P < 0.001$). Results showed an increase by 40.04–62.987%, depending on the dose, in comparison with the non-irradiated control.

To identify the groups between which these differences are found, and to ascertain the degree of significance for each comparison, the Tukey–Kramer *post-hoc* test was performed. The result showed very significant ($P < 0.010$) differences between the following groups: 0 Gy vs. 300 Gy and 400 Gy; 100 Gy vs. 200, 300, and 400 Gy. Significant ($P < 0.050$) differences were found between the 0 Gy vs. 200 Gy groups.

The total phenolic content of the fermented broths within and between irradiated groups is represented in Fig. 5.

Table 4. The one-way ANOVA of total phenolic content of the mycelium extracts

Groups	Count	Sum	Average	Variance	Std. deviation	Std. error
0 Gy	5	439.4212	87.8842	98.0936	9.9042	4.4293
100 Gy	4	451.3970	112.8492	124.0126	11.1361	5.5680
200 Gy	5	632.8615	126.5723	76.2878	8.7343	3.9061
300 Gy	5	684.2167	136.8433	417.0659	20.4222	9.1331
400 Gy	5	667.4257	133.4851	238.70581	15.4501	6.9095

ANOVA single factor ($\alpha = 0.01$)

Source of variation	SS	df	MS	F	P-value	F crit
Between groups	7 904.44478	4	1 976.111195	10.16779312	0.00014191	4.500257699
Within groups	3 692.651123	19	194.3500591			
Total	11 597.0959	23				

SS, sum of squares; df, degrees of freedom; MS, mean square.

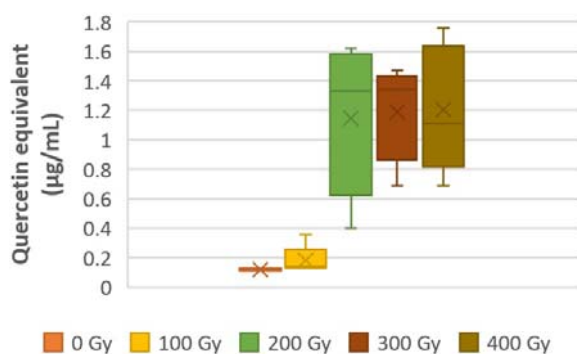


Fig. 5. The radiation dose-dependent distribution of total phenolic content of the fermented broths.

Determination of the total flavonoid content

The total flavonoid content of the crude culture broth increased by 26.6–934.678%, depending on the dose, in comparison with non-irradiated controls.

The statistical significance between the irradiated groups in terms of total flavonoid content of the fermented broths was evaluated by analysis of variance (Table 6). The result demonstrates that the differences are statistically highly significant ($F = 14.089$ and $P < 0.001$).

To identify the groups between which these differences are found, and to ascertain the degree of significance for each comparison, the Tukey–Kramer

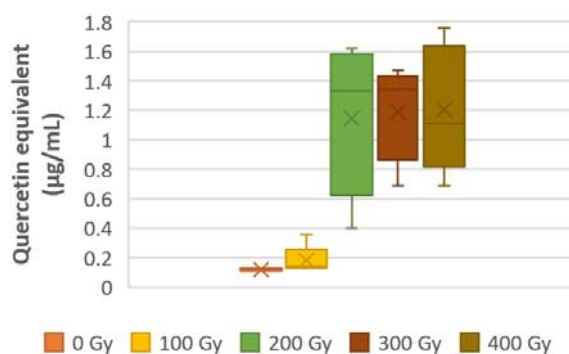


Fig. 6. The radiation dose-dependent distribution of total flavonoid content of the fermented broths.

post-hoc test was performed. The result showed very significant ($P < 0.010$) differences between the following groups: 0 Gy vs. 200, 300, and 400 Gy; 100 Gy vs. 200, 300, and 400 Gy.

The total flavonoid content of the fermented broths within and between irradiated groups is represented in Fig. 6.

Capillary electrophoresis analysis of excreted compounds

The concentrated fermentation broths were assessed by capillary electrophoresis using 14 standards, such as were rutin, naringenin, isoquercitrin, umbellifer-

Table 5. The one-way ANOVA of total phenolic content of the fermented broths

Groups	Count	Sum	Average	Variance	Std. deviation	Std. error
0 Gy	5	73.6308	14.72610	4.2755	2.0678	0.9247
100 Gy	5	72.1087	14.42170	0.4407	0.6639	0.2969
200 Gy	5	103.1155	20.62311	7.9304	2.8161	1.2594
300 Gy	5	111.8099	22.36199	3.9634	1.9908	0.8903
400 Gy	5	120.0085	24.00170	15.6380	3.9545	1.7685

ANOVA single factor ($\alpha = 0.01$)

Source of variation	SS	df	MS	F	P-value	F crit.
Between groups	389.6157862	4	97.403946550	15.10220151	0.000007715	4.430690161
Within groups	128.9930431	20	6.449652157			
Total	518.6088293	24				

SS, sum of squares; df, degrees of freedom; MS, mean square.

Table 6. The one-way ANOVA of total flavonoid content of the fermented broths

Groups	Count	Sum	Average	Variance	Std. deviation	Std. error
0 Gy	5	0.5825	0.1165	0.00007	0.008539	0.003819
100 Gy	5	0.9134	0.1826	0.00974	0.098740	0.044158
200 Gy	5	5.7376	1.1475	0.26596	0.515721	0.230637
300 Gy	5	5.9381	1.1876	0.10480	0.323743	0.144782
400 Gy	5	6.0272	1.2054	0.18652	0.431886	0.193145

ANOVA single factor ($\alpha = 0.01$)

Source of variation	SS	df	MS	F	P-value	F crit.
Between groups	6.392584426	4	1.598146107	14.08987153	0.000012734	4.430690161
Within groups	2.268503447	20	0.113425172			
Total	8.661087873	24				

SS, sum of squares; df, degrees of freedom; MS, mean square.

Table 7. The one-way ANOVA of the sinapinic acid content of the fermentation broths

Groups	Count	Sum	Average	Variance	Std. deviation	Std. error
0 Gy	5	40.0005	8.0001	25.7390	5.073365	2.536682
100 Gy	3	51.3022	17.1007	26.4171	5.139765	3.634363
200 Gy	2	58.7856	29.3928	304.6469	20.619987	14.580533
300 Gy	3	91.1330	30.3776	18.1002	4.254438	3.008342
400 Gy	1	55.2323	55.2323	0	0	0

ANOVA single factor ($\alpha = 0.01$)

Source of variation	SS	df	MS	F	P-value	F crit.
Between groups	2466.721606	4	616.6804015	11.17539311	0.001532718	6.422085458
Within groups	496.6378863	9	55.18198737			
Total	2963.359492	13				

SS, sum of squares; df, degrees of freedom; MS, mean square.

one, cinnamic acid, chlorogenic acid, sinapinic acid, ferulic acid, kaempferol, luteolin, coumaric acid, quercetol, caffeic acid, and gallic acid.

Electrophoresis of the samples showed the presence of several compounds in the culture broths. However, not all of the samples could be analysed due to a clogging issue with the capillaries when migrating the extracts.

Naringenin was found at a concentration ranging from 4.94 mg/100 mL to 15.16 mg/100 mL of broth, across all the irradiated groups. After applying an analysis of variance, no statistical significance was found ($F = 1.404$ and $P > 0.100$) linking the given radiation dose and increases in broth concentration of naringenin.

Isoquercitrin was found at a concentration ranging from 18.76 mg/100 mL to 131.27 mg/100 mL of broth, across all the irradiated groups. After applying an analysis of variance, no statistical significance was found ($F = 2.044$ and $P > 0.100$) linking the given radiation dose and increases in broth concentration of isoquercitrin.

Chlorogenic acid was found at a concentration ranging from 3.96 mg/100 mL to 7.21 mg/100 mL of broth, across all the irradiated groups. After applying an analysis of variance, no statistical significance was found ($F = 0.004$ and $P > 0.100$) linking the given radiation dose and increases in broth concentration of chlorogenic acid.

Luteolin was found at a concentration ranging from 2.15 mg/100 mL to 19.75 mg/100 mL of broth, across all the irradiated groups. After applying an analysis of variance, no statistical significance was found ($F = 2.409$ and $P > 0.100$) linking the given radiation dose and increases in broth concentration of luteolin.

Sinapinic acid was found at a concentration ranging from 1.41 mg/100 mL to 55.23 mg/100 mL of broth, across all the irradiated groups. After applying an analysis of variance, we found differences that are statistically highly significant (Table 7).

The sinapinic acid content of culture broth showed a 113.756–590.395% increase, depending on the dose, compared to the non-irradiated control.

To identify the groups between which these differences are found, and to ascertain the degree of significance for each comparison, the Tukey–Kramer

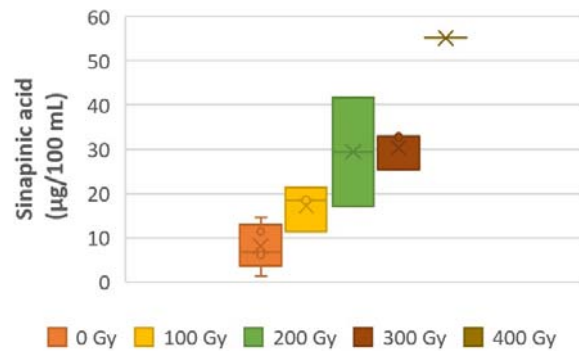


Fig. 7. The radiation dose-dependent distribution of sinapinic acid content of the fermentation broths.

post-hoc test was performed. The result showed very significant ($P < 0.010$) differences between the 0 Gy vs. 400 Gy groups. Significant ($P < 0.050$) differences were also found between the 0 Gy vs. 200 Gy and 300 Gy; 100 Gy vs. 400 Gy groups.

The total sinapinic acid content of the fermentation broths within and between irradiated groups is represented in Fig. 7.

Conclusion

In this study, oxidative stress induced by gamma irradiation on the mycelial inoculum of the medicinal fungus *I. obliquus*, followed by shake flask cultivation, has shown a very significant increase in the free radical scavenging activity and levels of total polyphenols, in the methanolic mycelium extracts. The optimal dose, according to these assayed criteria, is 300 Gy.

The fermentation broth of the cultures has shown a very significant increase in levels of total polyphenols and total flavonoids, corresponding to larger radiation doses. The optimal dose, in these instances, is 400 Gy. The broths were further assayed using capillary electrophoresis. Naringenin, isoquercitrin, chlorogenic acid, luteolin, and sinapinic acid were found to be present, but only the latter showed statistically significant increases correlated to higher radiation doses; in this case, the optimal dose was 300 Gy.

The total dry weight of the submerged cultured mycelia also showed a very significant increase after irradiation, with an optimal dose at 300 Gy

in one experiment, and 400 Gy in another. Both experiments show a trend of higher mycelial mass correlated with increasing irradiation.






To summarize, the 300–400 Gy dose interval proved optimal for inoculum pre-treatment in a biotechnological process of *I. obliquus* submerged cultivation, in order to obtain a synergic effect on biomass and antioxidant properties. A 16 days cultivation period, supporting a fivefold multiplication of the inoculum's volume, is an efficient reaction window to develop and reveal the stimulation effect.

Other authors used oxidative stress to trigger the enhanced synthesis of diverse secondary metabolites synthesis. Zheng *et al.* [6] increased the production of phenolic compounds in *I. obliquus* by imposing oxidative stress caused by addition of H₂O₂ and/or arbutin. Similarly, Zheng *et al.* [2] evidenced the involvement of nitric oxide (NO) in modulating metabolism of polyphenols by the fungus *I. obliquus* under submerged culture conditions. The photosynthetic efficiency markedly decreased in gamma-irradiated *Zygnema* spp. alga, from 1 kGy to 5 kGy, whereas antioxidant capacity significantly increased [13].

To our knowledge, this is the first work showing the use of sublethal doses of gamma irradiation as a pre-treatment for increasing biomass yield and for upregulating synthesis of biologically active metabolites in medicinal fungi. Since the treatment is applied to a small mycelium mass that is subsequently multiplied, such treatment is of particular interest for both fundamental biological processes and industrial potential. Medically potent compounds synthesis is yet to be investigated through this novel production system. The technique is both economically and environmentally effective in exploitation of endangered species and satisfying nutraceutical and pharmaceutical markets' demands for higher amounts of bioactive ingredients.

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